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14. ABSTRACT The ARF tumor suppressor protein plays an important role in the tumor surveillance of human cancer. In the search for novel ARF binding proteins, we uncovered NPM. Despite the important role ARF plays in the regulation of tumorigenesis, alterations selectively affecting its ability to negate NPM function have not been studied. In our proposed study, we aimed to determine the impact of ARF-NPM interactions in the pathogenesis of breast cancer. To this end, we have found that overexpression of NPM in the absence of ARF is a powerful transforming event. NPM promotes tumorigenesis without affecting genomic stability, implying that the subsequent tumors should remain diploid, a hallmark of ARF-null breast cancers. Indeed, when we analyzed sixty breast carcinomas, NPM was highly overexpressed in 50% of cases. We have begun further analyses of how NPM promotes tumor formation and have discovered that it does so through ribosome dysregulation, opening up the door to new therapeutic targets in breast cancer: protein synthesis.								
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INTRODUCTION

As the most prominent of subnuclear structures, the nucleolus has long been recognized as the site for active transcription of ribosomal RNAs (rRNA) and ribosome assembly (6). Various nucleolar proteins, RNAs, and other factors have been suggested to be involved in this complex process of ribosome production and maturation (10). Recently, several groups reported the successful isolation and mapping of the mammalian nucleolar proteome (1, 2, 20). While the nucleolar proteome contains many proteins and ribonucleoproteins proposed to be involved in ribosome biogenesis, a remarkable number of proteins identified (>100) have no known function. The difficulty in assessing nucleolar protein function stems from early assumptions that all nucleolar proteins must be involved, in some way, with static ribosome biogenesis by virtue of their unique subcellular localization. However, a more contemporary view of the nucleolus as a dynamic nuclear organelle capable of regulating numerous cellular processes has led to a re-evaluation of nucleolar protein function(s) (14).

The ARF tumor suppressor is localized to nucleoli in mammalian cells and plays an important role in preventing tumor development. Our initial studies have focused on identifying targets for ARF tumor suppression. One such target, NPM, was recently identified by our lab. Nucleophosmin (NPM/B23) is an abundant phosphoprotein localized in the granular regions of the nucleolus (22). NPM was found to be highly expressed in proliferating cells (7, 8), and has been associated with a variety of cellular phenomena, including ribosomal biogenesis, protein chaperoning and centrosome duplication (8, 13, 18, 19). Structurally, NPM can exist in both a monomeric and multimeric state, although NPM multimers seem to dominate in the nucleolus and may be crucial for the assembly of maturing ribosomes (16, 17, 24). More importantly, NPM, along with other nucleolar proteins, has been suggested to actively mobilize into distinct subcellular pools, supporting the notion that NPM trafficking may contribute to some of its essential functions (4). Indeed, NPM exit from the nucleolus/nucleus is an essential event in S phase progression; inhibition of this trafficking by the nucleolar tumor suppressor ARF results in cell cycle arrest (5). Additionally, NPM is an essential nucleolar protein with loss of its expression resulting in severe attenuation of cellular proliferation and increased apoptosis (3, 5, 9, 11), underscoring NPM's importance to the cell.

If nuclear exit of NPM plays a positive role in promoting cell growth and proliferation, what necessary function is it performing? While numerous proteins, such as Mdm2, cdc14p and TERT, are topologically restrained in the nucleolus following defined cellular cues, synthesis and export of newly synthesized ribosomal subunits from the nucleolus remains the only known nucleolar-specific event conserved throughout evolution (21). Recent work from *Xenopus laevis* and *Saccharomyces cerevisiae* has shown that nuclear export of ribosomes utilizes the CRM1-RanGTP export receptor pathway (12) as well as a nuclear adaptor protein NMD3 that is conserved from yeast to man (23).

Despite the seemingly important role ARF plays in breast tumor prevention, with over half of all breast cancers lacking ARF expression, studying the interplay between ARF and its targets, like NPM, has remained a largely unexplored theme. In my original proposal, I aimed to use a variety of molecular and genetic methods to more accurately address the broad question of how ARF restrains breast cancer progression.

BODY

Nucleophosmin (NPM/B23) is a key regulator in the regulation of a number of processes including centrosome duplication, genomic integrity and ribosome biogenesis. While the mechanisms underlying NPM function are largely uncharacterized, NPM loss results in severe dysregulation of developmental and growth-related events. We show that NPM utilizes a conserved CRM1-dependent nuclear export sequence in its amino-terminus to enable its shuttling between the nucleolus/nucleus and cytoplasm. In search of NPM trafficking targets, we biochemically purified NPM-bound protein complexes from HeLa cell lysates. Consistent with NPM's proposed role in ribosome biogenesis, we isolated ribosomal protein L5 (rpL5), a known chaperone for the 5S ribosomal RNA. Direct interaction of NPM with rpL5 mediated the co-localization of NPM with maturing nuclear 60S ribosomal subunits, as well as newly exported and assembled 80S ribosomes and polysomes. Inhibition of NPM shuttling or loss of *NPM* blocked the nuclear export of rpL5 and 5S rRNA, resulting in cell cycle arrest and demonstrating that NPM and its nuclear export provide a unique and necessary chaperoning activity to rpL5/5S (See attached paper, Yu et. al. 2006).

The nucleolus, a highly specialized and structured organelle, has been described as the cell's control center for ribosomal synthesis, maturation and assembly, with a host of proteins, RNAs and other factors being implicated in these processes. Recently, numerous proteins (cdc14, NPM, cyclin E, Mybbp1a, TERT and others) have been shown to continuously shuttle from the nucleolus to various subcellular compartments in a regulated manner, providing evidence that the nucleolus is a dynamic site of multiple cellular events.

One such protein, NPM/B23, has been linked to a variety of important cellular processes, both in and out of the nucleolus, including ribosome processing, molecular chaperoning, genomic integrity, centrosome duplication and transcriptional regulation. Initially, NPM which was imported into the nucleolus from the cytoplasm was presumed to move about the various compartments of the nucleus, a feature shared by many critical cell cycle regulators. This shuttling of proteins between the nucleus and cytoplasm is now recognized as a key mechanism for ensuring proper cell cycle progression. In previous reports, we and others identified NPM as a novel p53-independent target of the ARF tumor suppressor protein. We have since shown that, in response to hyperproliferative signals, nucleolar ARF directly binds NPM, effectively inhibiting NPM's nucleocytoplasmic shuttling. Here, we have further explored the mechanism and significance of NPM intracellular trafficking. First, we have described the CRM1-dependent nuclear export of NPM, identifying the two leucine residues (42 and 44) that are critical to this process. In addition, we have shown that alteration of the NPM NES resulted in the failure of wild-type NPM to be exported out of the nucleolus, providing

evidence that these mutations function in a dominant-negative fashion, through the formation of NPM-NPMdL hetero-multimers. Thus, NPMdL mimics the effects of ARF induction by directly impeding the nucleocytoplasmic shuttling of NPM through direct interaction, further demonstrating that NPM must exit the nucleolus/nucleus to maintain and promote cell growth.

We have previously proposed that targets of nucleolar sequestration might in fact “ride the ribosome” from the nucleolus to the cytoplasm to engage in growth promoting events. In agreement with this hypothesis, our findings reveal a direct interaction between NPM and rpL5, providing the first physical link between NPM and ribosomal subunits. Much of the fields’ focus has been on the putative role of rpL5 in delivering 5S rRNA to the nucleolus, following the initial transcription of 5S rRNA by RNA polymerase III in the nucleoplasm. However, it is also possible that rpL5 is a critical player in the export of the large ribosomal subunit (60S), containing 5S rRNA, from the nucleolus/nucleus to the cytoplasm after its assembly. Clearly, these latter events would render themselves sensitive to NPM regulation, given that NPM provides the necessary export signals and chaperoning capabilities (via rpL5) required to transport components of the ribosome to the cytosol. Indeed, inhibition of NPM nuclear export via deletion or mutation of its NES prevented the trafficking of rpL5, an integral component of the 60S ribosomal subunit. Moreover, reduction of NPM expression through RNA interference completely abolished the cytosolic stores of rpL5, underscoring the absolute requirement for NPM in rpL5 nuclear export. Thus, our initial hypothesis of “riding the ribosome” should be revised to “taking the ribosome for a ride”.

While many components of the ribosome, including rpL5, encode their own NES, it is clear that a single NES forms a relatively weak interaction with CRM1, suggesting a requirement for additional NESs in the efficient export of complexes. Consequently, proteins like NPM and NMD3 may have evolved to serve this purpose. Additionally, NPM and rpL5 were found, in reduced amounts, in cytosolic 40S and 60S complexes, respectively, after LMB treatment implying that either these particular protein-ribosome complexes are fairly stable or that a minor fraction of NPM and rpL5 utilize CRM1-independent modes of transport from the nucleus. Considering that the predominant function of rpL5 is to bind and mobilize 5S rRNA molecules, it was not surprising that 5S transport was also NPM-sensitive, and thus NPM contributes to the efficient nuclear export of rpL5-5S rRNA complexes. However, NPM was present in 40S, 60S, 80S and polysomes in the cytoplasm, implying that NPM, free (within the 40S subunit) or bound to rpL5, remains associated with the mature ribosome as it assembles and forms actively translating polysomes in the cytosol. Taken together, these findings open up the possibility that NPM might transmit additional cues (beyond nuclear export) to cytosolic ribosomes during translation, consistent with nucleolus’ proposed role in dictating translation rates.

While it has been appreciated for several decades that changes in nucleolar structure are reliable markers of cellular transformation, experiments that provide a direct link between nucleolar dysfunction and tumorigenesis remain to be conducted. In fact, the nucleolus has largely been dismissed as a static organelle, having little-to-no impact on the overall well-being of the cell. However, this “nucleolar stigma” recently has been challenged with the discovery that tumor suppressors, such as p53 and ARF, play a direct role in regulating nucleolar processes. Interestingly, rpL5 is also a binding partner of

Mdm2 and p53, suggesting that rpL5 may provide an intriguing mechanistic link between ARF and ARF-binding partners. Clearly, through its interaction with NPM, ARF is capable of inhibiting nuclear export of rpL5-5S rRNA complexes. Inhibition of NPM-directed rpL5-5S nuclear export by ARF or NPM defective shuttling mutants results in cell cycle arrest, demonstrating the importance of rpL5-5S export in maintaining cell proliferation. Moreover, NPM itself is a unique player in both the p53 and ARF responses, providing us with a glimpse of how this network of protein interactions may inevitably lend itself sensitive to oncogenic and tumor suppressive signals in determining tumorigenic cell fates.

Cellular growth (macromolecular synthesis) must be coupled to cell proliferation for proper transit through the cell cycle. The factors underscoring cell cycle control have been well studied. However, our knowledge of mechanisms that control cell growth in response to environmental cues is lacking. The nucleolus is at the center of growth sensing; it is the site of ribosome assembly, with nucleolar nucleophosmin (NPM) and p19ARF proteins antagonizing one another to either promote or inhibit growth, respectively. While ARF vigorously responds to hyperproliferative signals to shunt growth, we first noticed that nucleoli from *Arf*-/- MEFs displayed increased nucleolar area, suggesting that ARF might regulate key nucleolar functions in a pre-malignant cell. Ultrastructural analysis of *Arf*-/- nucleoli revealed increased irregularity and larger, more numerous fibrillar centers. In accord with these dysmorphic nucleoli, ribosomal content and total protein synthetic rates were dramatically elevated in the absence of *Arf*. Similar results were obtained using targeted lentiviral RNA interference of *Arf* in wild-type cells, further implicating basal ARF proteins in the regulation of nucleolar structure and function. Finally, *Arf*-/- osteoclasts, post-mitotic cells whose activities are intimately tied to their protein synthesis rates, exhibited enhanced differentiation and resorptive functions, demonstrating a physiological function for ARF in maintaining proper basal protein synthesis *in vivo*. Taken together, these data indicate that disruption of *Arf* greatly impacts ribosomal biogenesis and translational control, providing a significant teleological role for ARF as a monitor of cellular growth independent of its ability to prevent unwarranted cell cycle progression (see attached manuscript, Apicelli et. al, 2007).

KEY RESEARCH ACCOMPLISHMENTS

- NPM shuttles rpL5 to the cytosol
- NPM actively shuttles rRNA from the nucleolus to the cytoplasm
- ARF inhibits NPM shuttling
- ARF inhibits rRNA nuclear export
- Loss of *Arf* results in tremendous gains in rRNA synthesis
- Loss of *Arf* causes severe changes in nucleolar morphology
- Osteoclasts lacking *Arf* exhibit amplified protein synthesis rates
- Basal ARF proteins have a role in regulating the homeostasis of the nucleolus

- “Nucleophosmin is Essential for Ribosomal Protein L5 Nuclear Export”, Yu et al. *Molecular and Cellular Biology* (2006) **26**:3798-3809.
- “Therapeutic Targets in the ARF Tumor Suppressor Pathway”, Saporita et al. Submitted (2007).
- “A Non-Tumor Suppressor Role for p19ARF in Maintaining Nucleolar Structure and Function”, Apicelli et al. Submitted (2007).

REPORTABLE OUTCOMES

- “Nucleophosmin is Essential for Ribosomal Protein L5 Nuclear Export”, Yu et al. *Molecular and Cellular Biology* (2006) **26**:3798-3809.
- “Therapeutic Targets in the ARF Tumor Suppressor Pathway”, Saporita et al. Submitted (2007).
- “A Non-Tumor Suppressor Role for p19ARF in Maintaining Nucleolar Structure and Function”, Apicelli et al. Submitted (2007).
- “The Role of ARF in Nucleolar Dynamics” Poster Presentation. Meeting on the Nucleolus: Structure and Function, Oxford, England (2006).
- Experience in generating and breeding transgenic mice

CONCLUSIONS

This proposal was designed to investigate the opposing roles of ARF and NPM in the pathogenesis of breast cancer. In the first year of support, we have generated a significant amount of data that should help our lab and others understand the intricate mechanism(s) by which ARF targets NPM to suppress tumor formation. Additionally, we now know that NPM is overexpressed in human breast carcinomas and that in this context, it is a potent oncogene. We have two published papers on the significance of the ARF-NPM interaction and have submitted another manuscript that discusses a proposed role for ARF in maintaining proper ribosome biogenesis. The latter could be quite important as it opens the door to a whole new array of putative anti-cancer targets that might be involved in protein translation. We have begun work on the last specific aim, having generated a colony of transgenic mice that overexpress NPM in the breast epithelium. We are looking forward to another productive year working on this important project.

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APPENDICES

1. “Nucleophosmin is Essential for Ribosomal Protein L5 Nuclear Export”, Yu et al. *Molecular and Cellular Biology* (2006) **26**:3798-3809.
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Nucleophosmin Is Essential for Ribosomal Protein L5 Nuclear Export

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Nucleophosmin (NPM/B23) is a key regulator in the regulation of a number of processes including centrosome duplication, maintenance of genomic integrity, and ribosome biogenesis. While the mechanisms underlying NPM function are largely uncharacterized, NPM loss results in severe dysregulation of developmental and growth-related events. We show that NPM utilizes a conserved CRM1-dependent nuclear export sequence in its amino terminus to enable its shuttling between the nucleolus/nucleus and cytoplasm. In search of NPM trafficking targets, we biochemically purified NPM-bound protein complexes from HeLa cell lysates. Consistent with NPM's proposed role in ribosome biogenesis, we isolated ribosomal protein L5 (rpL5), a known chaperone for the 5S rRNA. Direct interaction of NPM with rpL5 mediated the colocalization of NPM with maturing nuclear 60S ribosomal subunits, as well as newly exported and assembled 80S ribosomes and polysomes. Inhibition of NPM shuttling or loss of NPM blocked the nuclear export of rpL5 and 5S rRNA, resulting in cell cycle arrest and demonstrating that NPM and its nuclear export provide a unique and necessary chaperoning activity to rpL5/5S.

As the most prominent of subnuclear structures, the nucleolus has long been recognized as the site of active transcription of rRNA and ribosome assembly (8). Various nucleolar proteins, RNAs, and other factors have been implicated in the complex process of ribosome production and maturation (18). Recently, several groups reported the successful isolation and mapping of the mammalian nucleolar proteome (1, 2, 44). While these studies clearly identified proteins and ribonucleoproteins with purported roles in ribosome biogenesis, a surprising number of proteins within the nucleolar proteome (>100) have no known function. In previous decades, it was assumed that all nucleolar proteins must somehow contribute to static ribosome biogenesis simply by virtue of their localization. However, more-recent findings have demonstrated that the nucleolus is a dynamic subnuclear organelle which regulates numerous cellular processes, prompting a broadened view of the potential functions of nucleolar proteins (28).

Nucleophosmin (NPM/B23) is an abundant phosphoprotein that resides within the granular regions of the nucleolus (46). Proliferating cells express NPM at high levels (9, 13), and NPM has been associated with a variety of cellular events, including ribosomal biogenesis, protein chaperoning, and centrosome duplication (13, 23, 35, 36). Structurally, NPM is present in both monomeric and multimeric states, although NPM multimers appear predominant in the nucleolus and may be crucial for the assembly of maturing ribosomes (33, 34, 53). Furthermore, NPM, along with other nucleolar proteins, is believed (or has been shown) to actively mobilize into distinct subcellular pools, supporting the notion that NPM trafficking may be

essential for its (proper) function (6). Indeed, NPM's transit from the nucleolus/nucleus is an essential event in S phase progression; when NPM export was inhibited by the nucleolar tumor suppressor ARF, cells arrested in G₁ (7). Moreover, loss of NPM expression results in severe attenuation of cellular proliferation and increased apoptosis (5, 7, 16, 19), underscoring NPM's indispensable role within the cell.

Given that nuclear export of NPM promotes cell growth, we aimed to further elucidate the crucial roles of NPM's trafficking. While numerous proteins, such as Mdm2, cdc14p, and telomerase reverse transcriptase, are topologically restrained in the nucleolus following receipt of defined cellular cues, newly synthesized ribosomal subunits must be exported from the nucleolus to promote proper protein translation in the cytosol (45). Recent work with *Xenopus laevis* and *Saccharomyces cerevisiae* has shown that nuclear export of ribosomes utilizes the CRM1-RanGTP export receptor pathway (20) as well as the conserved nuclear adaptor protein NMD3 (51). While investigating the critical nature of NPM trafficking, we noted that NPM's exit from the nucleus also involved the classical CRM1-dependent nuclear export pathway. In search of proteins that are targeted for NPM-mediated nuclear export, we observed that nuclear and cytosolic NPM proteins directly bound to the ribosomal L5 protein (rpL5), a 60S subunit protein that chaperones the 5S rRNA into the nucleolus and out into the cytosol (31). Here we report that NPM mediates rpL5/5S nuclear export through a CRM1-dependent mechanism, allowing NPM to directly access the maturing ribosome and potentially regulate the protein translational machinery.

MATERIALS AND METHODS

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Cell culture. HeLa and NIH 3T3 cells and wild-type (WT) mouse embryonic fibroblasts (MEFs) (ArtisOptimus, Carlsbad, CA) were maintained in Dulbecco's

modified Eagle's medium with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, and 100 U penicillin and streptomycin.

Plasmid constructs. Vectors encoding full-length His-tagged murine NPM are described elsewhere (7). The His epitope-tagged NPM coding sequence was subcloned into pcDNA3.1 (Invitrogen) and pEGFP (Clontech) vectors. His-NPM_{Δ42–61}, His-NPM_{Δ62–83}, or His-NPMdL mutants were generated using the primers 5'-GAAAATGAGCACCGCAGAAGCAATGAAC-3' (sense) and 5'-GTTCTATTGCTCTGCCTGGTGCCTCATTTTC-3' (antisense), 5'-GTTACACATCGTAGCAACCAACAGTTCC-3' (sense) and 5'-GAAACTGTGTTGGTGTCTACGATGTAAAC-3' (antisense), or 5'-GAAAATGAGCACAGCGTCAGCAAGAACGGTC-3' (sense) and 5'-CTAAACTGACCGTTCTTGTGACGCCGGTGCCTCATTTTC-3' (antisense), respectively, by QuikChange mutagenesis (Stratagene). A myc-tagged NPC-M9 (40) in pcDNA3 and a green fluorescent protein (GFP)-tagged rpL5 plasmid (41) were generous gifts from Alan Diehl (University of Pennsylvania) and Joachim Hauber (Universität Erlangen-Nürnberg).

Heterokaryon assay. HeLa cells (2×10^5) were seeded onto glass coverslips and transfected with plasmids. NIH 3T3 cells (6×10^5) were seeded onto the HeLa cells 24 h posttransfection. Cocultures were then incubated for 30 min with cycloheximide (100 µg/ml), followed by incubation with 50% polyethylene glycol in phosphate-buffered saline for 105 s. Cocultures were incubated with Dulbecco's modified Eagle's medium containing cycloheximide (100 µg/ml) for an additional 4 h. Heterokaryons were fixed and stained with a rabbit anti-His antibody (Santa Cruz) or mouse anti-myc antibody (Zymed), followed by either fluorescein isothiocyanate-conjugated or rhodamine X-conjugated anti-rabbit or anti-mouse immunoglobulin (Pierce) as described previously (7). Nuclei were stained with Hoechst (Sigma). Fluorescent signals were detected using a Nikon epifluorescence compound microscope ($\times 100$) fitted with a Nikon FDX-35 camera.

Immunoprecipitation and Western blot analysis. Cells were transduced as recommended by the manufacturer (Amaxa) with vectors encoding His-NPM, His-NPMdL, and GFP-rpL5 and lysed in binding buffer (25 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40) 48 h after the Nucleofector process. Primary antibody to the NPM N terminus (custom rabbit; Sigma Genosys), GFP (Santa Cruz), His (Santa Cruz), rpL5 (12), or nonimmune rabbit serum (NRS) was added to the binding reaction mixtures. Immune complexes were precipitated with protein A-Sepharose (Amersham). The precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. NPM, His-tagged proteins, and GFP-tagged proteins were visualized by direct immunoblotting with NPM (Zymed), His (Santa Cruz), and rpL5 and GFP (Santa Cruz) antibodies, respectively.

Fluid phase liquid chromatography. For affinity chromatography, a rabbit polyclonal antibody recognizing the N terminus of NPM (Sigma) was coupled to *N*-hydroxysuccinimide-activated Sepharose (Amersham). HeLa cells were lysed in 20 mM Tris, pH 7.4, and 0.1% Tween 20 and sonicated. Lysates (600 µg) were injected onto the NPM affinity column, washed with 20 mM Tris, and eluted with an increasing NaCl gradient (0.1 to 1 M) using BioLogic fluid phase liquid affinity chromatography and HR software (Bio-Rad). Fractions were precipitated with trichloroacetic acid (TCA). Proteins were resuspended in 1 M Tris-HCl (pH 7.4), separated by SDS-PAGE, and visualized with Coomassie blue stain (Sigma).

Proteomic analysis. Proteins from fluid phase liquid affinity chromatography fractions were precipitated with TCA and resuspended in Laemmli buffer. SDS-PAGE-separated proteins were stained with SYPRO-Ruby (Bio-Rad). Bands of interest were excised and processed for trypsin digestion. Tryptic peptides were calibrated with Sequazyme peptide mass standard kit (PE Biosystem) and analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Voyager DE Pro; Applied Biosystems). Identification of proteins was performed using MS-Fit software (<http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm>). MALDI-TOF spectra and sequences were verified using a 4700 Proteomics tandem mass spectrometry system (Applied Biosystems). Identified proteins were additionally verified by direct Western blot analysis.

Bacterial protein purification. BL21 cells were transformed with pET28a vectors encoding NPM, NPMdL, rpL5, and p27kip1 proteins. Protein production was induced for 3 h with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Harvested cells were lysed in phosphate-buffered saline containing protease inhibitors and 1% Triton X-100 with sonication. Cleared lysates were subjected to affinity purification using Ni-nitrilotriacetic acid columns as described by the manufacturer (Sigma). Purified proteins were separated by SDS-PAGE and visualized for purity using Coomassie blue stain.

Subcellular fractionation. HeLa cells were subjected to the Nucleofector process with scrambled or small interfering NPM RNAs or control vector, His-NPM, and His-NPMdL and harvested. Pellets containing equal cell numbers were resuspended in HEPES buffer (10 mM HEPES, pH 7.4, with 4 mM MgCl₂, 1 mM

phenylmethylsulfonyl fluoride [PMSF], 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin) and lysed with a syringe. Lysates were pelleted, and the supernatant was saved as the cytoplasmic fraction. The pellet was resuspended in fractionation buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 4 mM MgCl₂, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin), subjected to Dounce homogenization, layered over a cushion of sucrose (45%, wt/vol, in fractionation buffer), and centrifuged. The pellet was washed and resuspended in EBC buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM NaF, 10 mM NaVO₄, β-glycerophosphate). Nuclear or cytoplasmic protein was subjected to SDS-PAGE. Superoxide dismutase (SOD; Cu/Zn-specific form), lamin A/C, and rpL5 proteins were visualized by direct immunoblotting with anti-SOD (Calbiochem), anti-lamin A/C (Santa Cruz), and anti-rpL5 antibodies (12), respectively. Similarly, total RNA was isolated from the fractions obtained above and separated on formaldehyde-agarose gels. Separated RNA from each nuclear and cytoplasmic fraction was analyzed by Northern blotting using a probe specific for the 5S rRNA. The 5S rRNA probe was obtained by PCR using HeLa cell genomic DNA as the template and the following primers: sense, 5'-CCTTCAGCGTCTACGGCCATACC-3'; antisense, 5'-GCCAAGAAAAGCCTACAGCAGG-3'. The PCR product was cloned and confirmed by sequencing.

RNA FISH. HeLa cells were subjected to the Nucleofector process with pcDNA3.1 His, His-tagged NPM, or His-NPMdL and plated on coverslips. Cells were subjected to RNA fluorescence *in situ* hybridization (FISH) as described previously (3) using a tetramethyl rhodamine isocyanate (TRITC)-labeled 5S rRNA probe (Genedetect). DNA was counterstained with DAPI (4',6'-diamidino-2-phenylindole).

Ribosome fractionation. Cells were subjected to cytosolic and nuclear ribosome fractionation, and lysates were separated on sucrose gradients as previously described (48). RNA was continuously monitored over the gradient by measuring UV absorbance at 254 nm. Fractions were collected, and proteins were precipitated with TCA. Proteins were separated by SDS-PAGE and immunoblotted with antibodies recognizing NPM and rpL5.

RESULTS

NPM nuclear export requires a CRM1-dependent nuclear export signal involving leucines 42 and 44. NPM is a ubiquitously expressed nucleolar phosphoprotein capable of regulated nuclear import (6). When NPM is transiently expressed in mammalian cells, it localizes predominantly to the nucleolus. Moreover, using *in vivo* heterokaryon shuttling assays (50), we have previously shown that NPM readily shuttles between the nucleolus/nucleus and cytoplasm (7). NPC-M9, a nuclear hnRNP protein that readily mobilizes to the cytoplasm, serves as a shuttling control (40). To distinguish between human donor and murine acceptor nuclei, chromosomal DNA was stained with Hoechst, clearly demarcating greater heterochromatin foci of NIH 3T3 mouse cells (speckled pattern, Fig. 1, Hoechst). As shown in Fig. 1A, NPM readily shuttles out of the human nucleolus, into the fused cytoplasm, and back into the mouse acceptor nucleus/nucleolus.

Given that a wide range of shuttling proteins utilize the CRM1 transport protein for their nuclear export, we further investigated the underlying export mechanism of NPM both in the presence and absence of leptomycin B (LMB), a potent inhibitor of CRM1-mediated nuclear export (24). In the absence of LMB, NPM readily migrated from human nucleoli to mouse nucleoli (Fig. 1A). However, in the presence of LMB, NPM failed to shuttle and was restricted to human nucleoli within heterokaryons (92% inhibition; Fig. 1B). The addition of LMB did not hinder the nucleocytoplasmic trafficking of Myc-NPC-M9, an hnRNP that readily shuttles in a CRM1-independent nuclear export pathway (38).

A sequence alignment of NPM residues with known CRM1-dependent shuttling proteins revealed two motifs containing

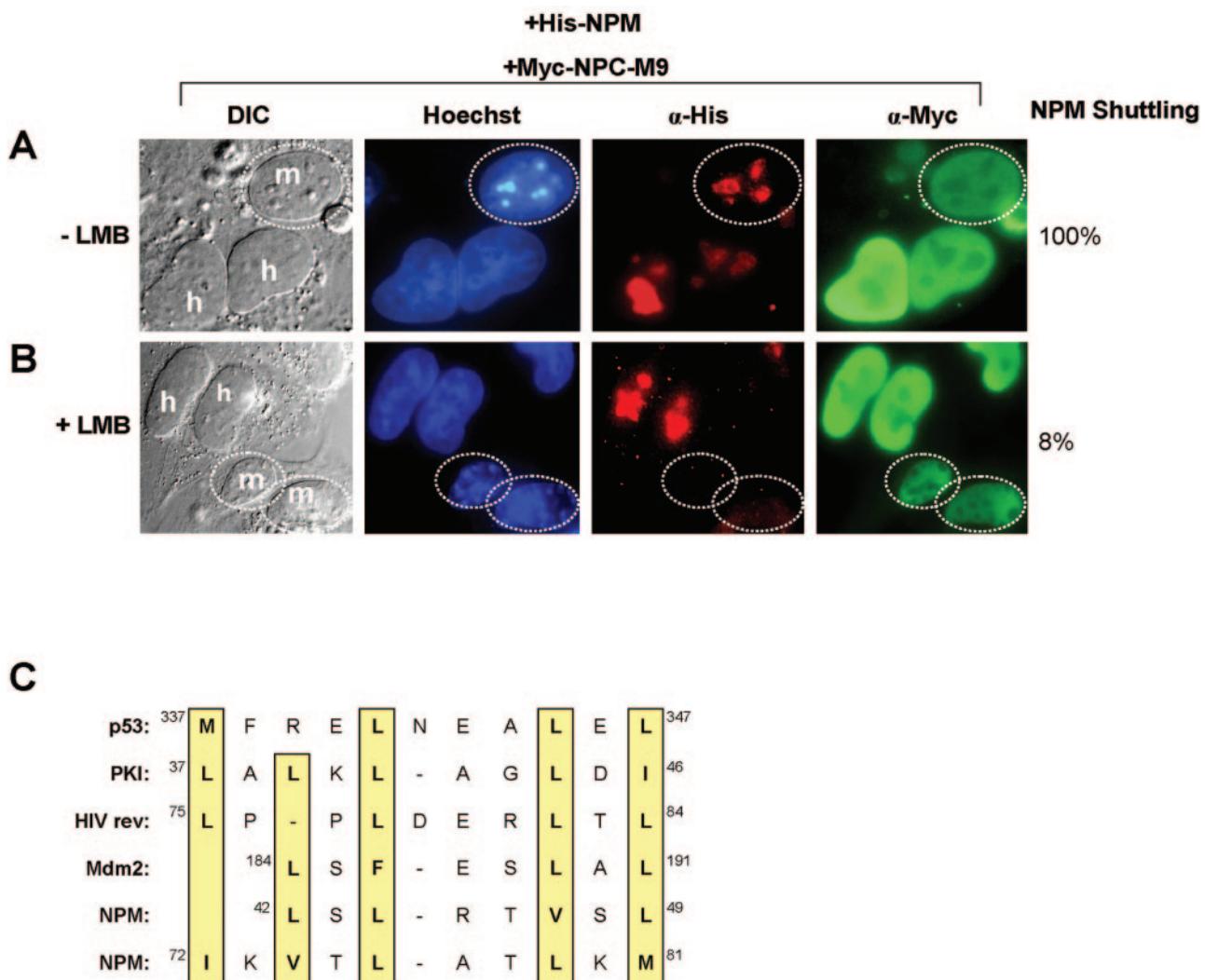


FIG. 1. Nuclear export of NPM is CRM1 dependent. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with His-NPM in combination with Myc-NPC-M9 (shuttling control) in the (A) absence or (B) presence of LMB. Heterokaryons were incubated in media containing cycloheximide for an additional 4 h before fixation. Heterokaryon formation was verified by phase-contrast microscopy, while His-NPM and Myc-NPC-M9 proteins were visualized with antibodies against His (red) and Myc (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments. The percentages of His-NPM shuttling in heterokaryons are given. DIC, differential interference contrast; α , anti. (C) Sequence alignment of putative NPM NESs with known NESs of CRM1-dependent nuclear export proteins (p53, protein kinase inhibitor [PKI], rev, and Mdm2). Critical hydrophobic residues are indicated in yellow.

short leucine-rich hydrophobic stretches of amino acids characteristic of CRM1-dependent nuclear export sequences (NESs) (Fig. 1C) (14, 15). In order to identify which region(s) of NPM contains its NES, we generated deletion mutants of NPM lacking either of the two potential NESs ($\text{NPM}_{\Delta 42-61}$ and $\text{NPM}_{\Delta 62-83}$). Using these NPM constructs, we again conducted interspecies heterokaryon assays. As shown in Fig. 2A, deletion of amino acids 42 to 61 of NPM ($\text{His-NPM}_{\Delta 42-61}$) prevented its shuttling (100% inhibition) to mouse nucleoli. Importantly, a myc-tagged NPC-M9 shuttling control readily shuttled in the same human-mouse heterokaryon, indicating that these heterokaryons were not impaired for nucleocytoplasmic shuttling in general. In contrast, deletion of amino acids 62 to 83 of

NPM ($\text{His-NPM}_{\Delta 62-83}$) did not prevent NPM from shuttling between human and mouse nucleoli (6% inhibition; Fig. 2B), revealing that the putative NES resides within amino acids 42 to 61 of the NPM protein.

Since the type of NES recognized and bound by the CRM1 export receptor is dependent on closely spaced hydrophobic amino acids (particularly leucines) (14, 15), we introduced point mutations into the corresponding leucine residues within the NES of NPM (Leu-42 and Leu-44 to Ala-42 and Ala-44). First, we tested this NPM mutant (designated NPM_{dL} for double-leucine mutant) with Myc-NPC-M9 as a shuttling control. As expected, NPM_{dL} was unable to transit from a human nucleus to the cytoplasm and into a murine nucleus (100%

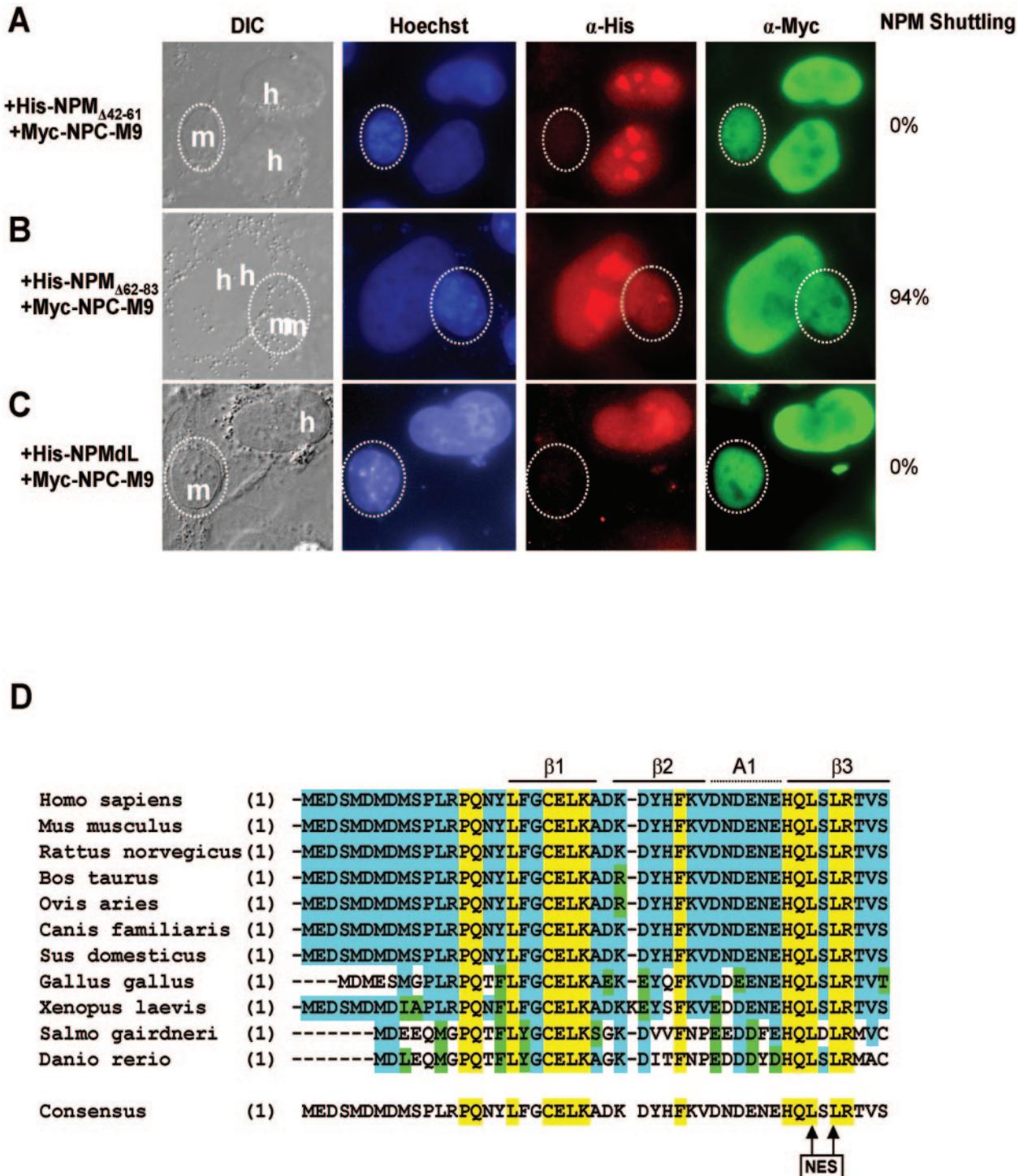


FIG. 2. Leucine 42 and leucine 44 are identified as critical nuclear export residues. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with (A) His-NPM_{Δ42-61}, (B) His-NPM_{Δ62-83}, or (C) NPMdL in combination with Myc-NPC-M9. Ectopic NPM proteins and Myc-NPC-M9 proteins were visualized with antibodies against His (red) and Myc (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments. The percentages of His-NPM shuttling in heterokaryons are given. DIC, differential interference contrast; α , anti. (D) Sequence alignment of NPM homologues throughout evolution. Identical residues in all species are marked yellow, identical residues in at least seven species are highlighted blue, and conserved residues are marked green. Crystal structure features are identified above the sequences. The consensus NPM sequence for all 11 identified homologues is given, with conserved nuclear export leucines 42 and 44 marked with arrows (NES).

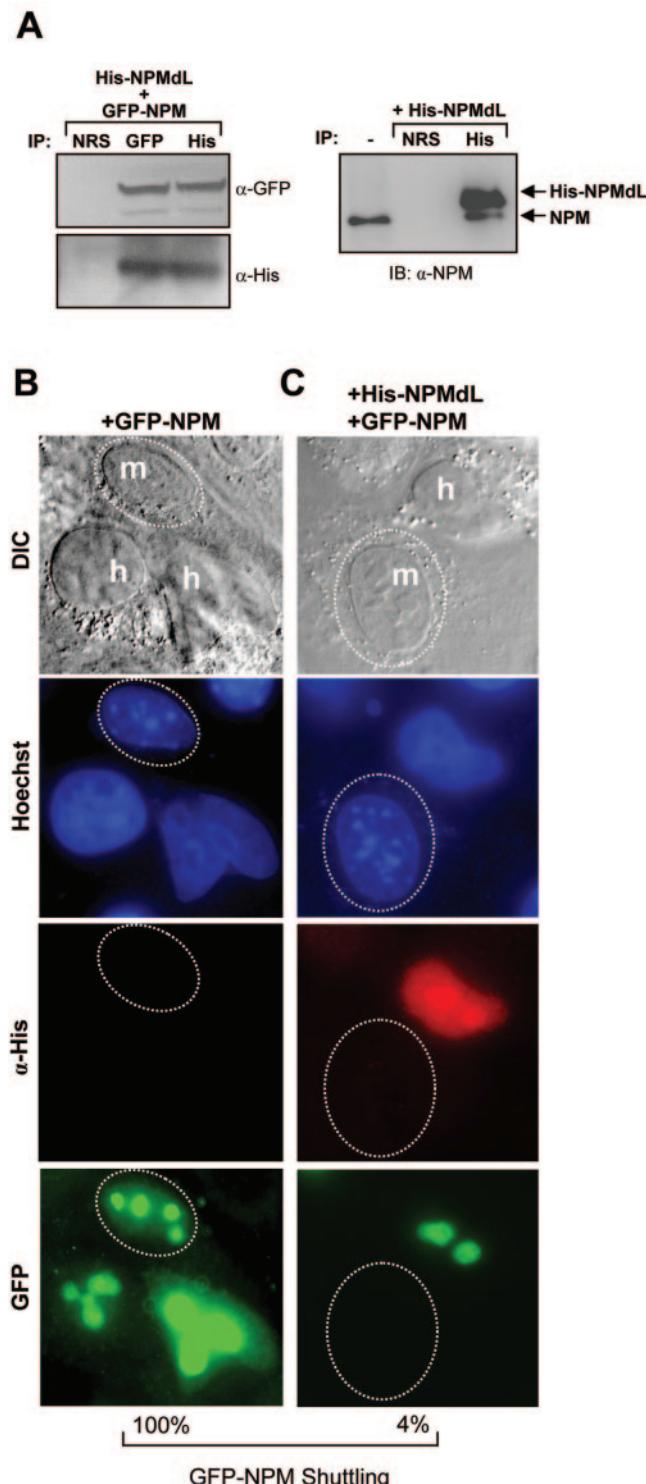


FIG. 3. NPM shuttling mutants act as dominant negative inhibitors of NPM nuclear export. (A, left) HeLa cells transduced with His-NPMdL and GFP-NPM were lysed, and the whole-cell lysate was subjected to immunoprecipitation (IP) with NRS or antibodies recognizing His and GFP epitopes. Precipitated protein complexes were separated by SDS-PAGE, and ectopic NPM proteins were visualized with antibodies against GFP and His epitopes. (A, right) HeLa cells transfected with His-NPMdL were lysed, and the whole-cell lysate was subjected to immunoprecipitation with NRS or antibodies recognizing His epitopes. Precipitated protein complexes were separated by SDS-

inhibition), indicating that these two leucine residues are critical for nuclear export of the NPM protein (Fig. 2C). Sequence alignment of numerous nucleophosmin homologues underscores the evolutionary importance of this amino-terminal export motif as it is nearly identical from zebra fish to humans (Fig. 2D).

Heterogeneous complexes containing NPM NES mutants and wild-type NPM fail to shuttle. Because NPM readily self-oligomerizes (32, 33, 34, 53), we considered the possibility that mutant NPM molecules could form hetero-oligomers with wild-type NPM proteins. To test this hypothesis, HeLa cells were transduced with His-tagged NPMdL expression vectors. Immunoprecipitation of His-NPMdL proteins revealed the co-precipitation of wild-type endogenous NPM proteins, demonstrating the formation of mutant-wild-type hetero-oligomers in cells (Fig. 3A, right). Additionally, cells transduced with His-NPMdL and GFP-NPM displayed formation of hetero-oligomers, as observed by coprecipitation of both proteins using antibodies directed at either epitope tag (His or GFP; Fig. 3A, left). Given our finding that mutant NPM forms oligomers with wild-type NPM, we next examined whether the NPM shuttling mutant NPMdL could also block wild-type NPM from shuttling. In the absence of the shuttling mutant, GFP-tagged NPM readily shuttled from human to mouse nucleoli (Fig. 3C). However, in the presence of His-tagged NPMdL, GFP-NPM was retained in human nuclei (Fig. 3D; 96% inhibition). Although we were unable to determine the exact stoichiometry between mutant proteins and wild-type proteins in the NPM oligomer, it is clear that overexpression of NPMdL severely impaired the shuttling activity of nearly all NPM oligomers.

NPM associates with cytoplasmic and nuclear rpL5 ribosome complexes. Previous studies have indicated that NPM might function as an integral component of ribosome maturation through its RNA binding activities (36). However, most hypotheses in this regard are largely based on the fact that NPM is nucleolar and, thus, most likely to be involved in the major process in the nucleolus: ribosome biogenesis. To formally test the nucleolar function of NPM, we examined the composition of in vivo NPM protein complexes in HeLa cell lysates. We generated a custom NPM polyclonal antibody affinity column and used a control nonimmune immunoglobulin column to preclear protein lysates. NPM complexes were eluted with increasing salt concentrations and visualized following SDS-PAGE and SYPRO-Ruby staining. As seen in Fig. 4A, we observed very little protein bound to our non-

PAGE, and ectopic mutant and endogenous wild-type NPM proteins were visualized with antibodies against NPM. Untransfected HeLa whole-cell lysate was loaded as a marker for endogenous NPM expression (lane 1). IB, immunoblot; α , anti; DIC, differential interference contrast. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with GFP-NPM (B) alone or (C) in combination with His-NPMdL. Heterokaryon assays were performed, and His-NPMdL and GFP-NPM proteins were visualized with antibodies against His (red) or naturally emitting GFP spectra (green). DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments. The percentages of GFP-NPM shuttling in heterokaryons are given.

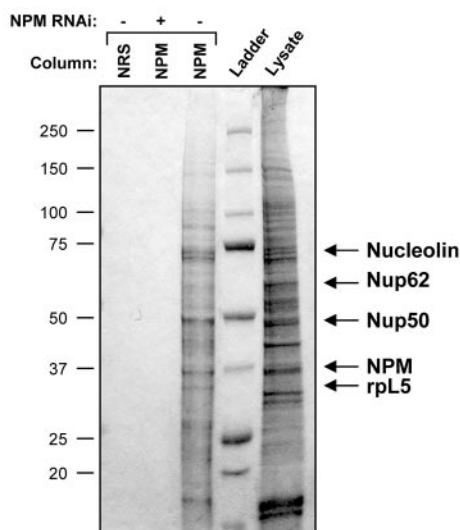
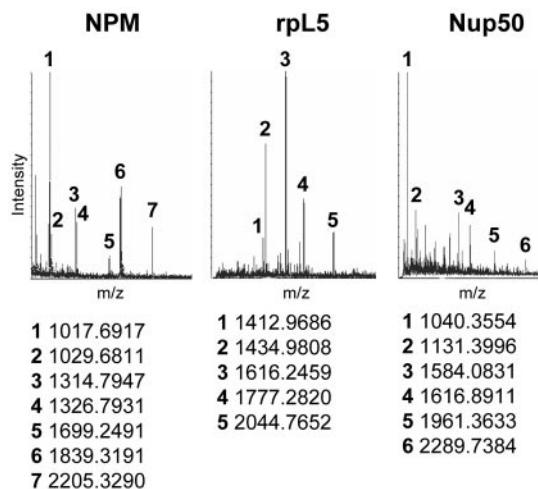
A**B**

FIG. 4. Isolation of endogenous NPM protein complexes. (A) HeLa cell lysates (600 µg) transduced with control (−) or NPM-directed RNA interference (RNAi) constructs (+) were injected onto either an NRS column or custom NPM polyclonal antibody affinity columns and eluted with an increasing NaCl gradient (0.1 to 1.0 M). Eluted proteins were separated by SDS-PAGE and visualized with Coomassie blue stain. Identified bands are labeled. (B) Representative MALDI-TOF spectra of labeled protein bands from panel A are shown with labeled matching peptide masses.

immune rabbit serum column (lane 1). However, some proteins (~18) were specifically eluted from the NPM antibody column (lane 3), including NPM and the previously known binding protein nucleolin. To determine whether the eluted proteins were in fact bound to the column through their interaction with NPM, we depleted NPM from HeLa cells using NPM-targeted RNA interference. Knockdown of NPM resulted in a loss of specific proteins bound and eluted from the

NPM antibody column, demonstrating that our identified NPM protein complex is specific for NPM (lane 2). Protein bands were excised and identified using MALDI-TOF and tandem mass spectrometry analyses. Among those proteins bound to NPM, a cluster of proteins associated with ribosome biogenesis, including rpL5 and nucleolin, as well as the nuclear pore complex proteins Nup50 and Nup62, were identified (Fig. 4A and B), with nucleolin (C23) being the only known NPM binding protein (26, 27). Western blot analysis of NPM protein complexes verified the presence of these proteins in salt-eluted fractions (data not shown).

Given the novelty and potentially significant ribosome biology of finding rpL5 in the NPM complex, we focused on verifying the NPM-rpL5 interaction. Purified recombinant NPM, NPMdL, and rpL5 proteins (Fig. 5A, left panels) mixed overnight were coprecipitated (Fig. 5A, middle panels), demonstrating that the NPM-rpL5 interaction is direct and independent of the NPM nuclear export signal. To show that the interaction of recombinant proteins was specific, NPM and rpL5 were mixed overnight with recombinant p27kip1 proteins (equally charged proteins not bound to the NPM antibody column). Precipitated proteins exhibited no complex formation between NPM and p27kip1 or rpL5 and p27kip1 (Fig. 5A, right panels). Both NPM and rpL5 readily interact with RNAs through conserved nucleic acid binding domains. To determine whether RNA binding is required for the NPM-rpL5 interaction, HeLa lysates were subjected to RNase A treatment prior to coprecipitation of NPM-rpL5 complexes. Even in the presence of RNase A, NPM and rpL5 visibly formed in vivo protein complexes (Fig. 5B) indistinguishable from those from untreated cells and consistent with our earlier finding that the interaction can be recapitulated with purified recombinant proteins (Fig. 5A). While NPM and rpL5 formed complexes in vivo, serial immunoprecipitation of NPM proteins from HeLa lysates showed that NPM and rpL5 are not exclusive partners. We failed to detect rpL5 in some NPM complexes (Fig. 5C, 3° and 4°), and we also noted that there was a significant amount of rpL5 free from NPM complexes in the remaining supernatant (Fig. 5C, Sup), indicating that both NPM and rpL5 can exist in complexes independent of one another.

Having identified a critical member of the 60S ribosomal subunit, namely, rpL5, in NPM complexes, we wanted to evaluate the colocalization of NPM with ribosomes in vivo. In order to follow the spatial control of NPM-rpL5 complexes in vivo, we utilized the UV absorbance of the ribosome. Ribosomal protein L5 is known to supply the maturing 60S ribosomal subunit with 5S rRNA prior to nucleolar/nuclear export of the 60S subunit (47), providing NPM an ideal time to form nucleolar complexes with rpL5. Cytoplasmic and nuclear extracts of HeLa cells were subjected to sucrose gradient centrifugation, and the gradients were fractionated with continuous UV monitoring. As shown in Fig. 6, NPM associates with the 40S, 60S, 80S, and polysome fractions in the cytoplasm while nuclear pools of NPM associate with the 40S/pre-60S and 60S fractions in the nucleus. Consistent with previous reports (30), we found rpL5 associated with the 60S, 80S, and polysome fractions in the cytoplasm and the 40S/pre-60S and 60S fractions in the nucleus (Fig. 6). These data demonstrate that NPM and rpL5 are localized with the maturing 60S ribosomal subunits in the nucleus and are maintained in the mature ribo-

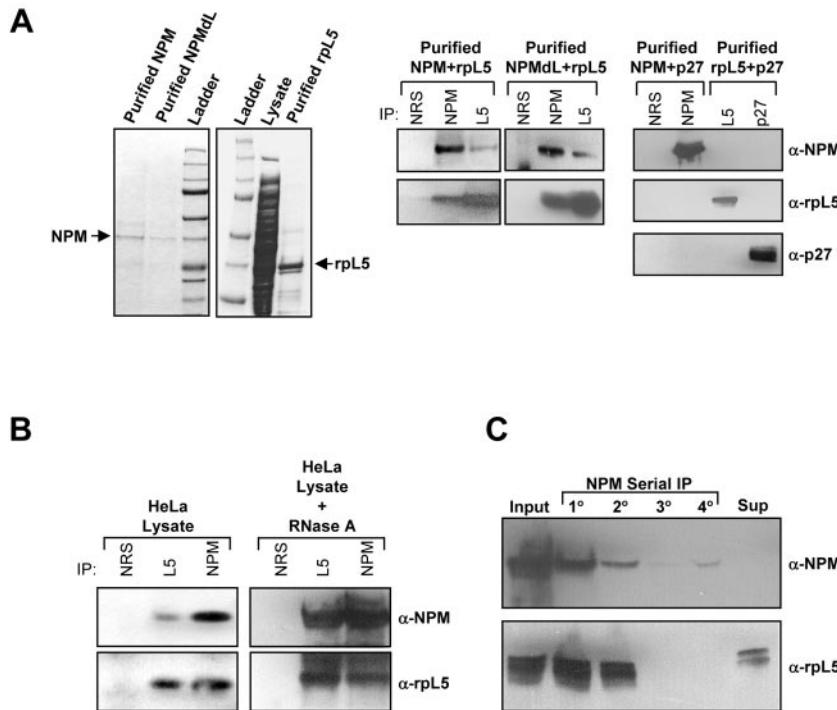


FIG. 5. NPM interacts directly with rpL5. (A, left) Recombinant NPM, NPMdL, and rpL5 were purified from bacterial lysates using Ni-nitrilotriacetic acid affinity chromatography. Purified proteins were separated by SDS-PAGE and detected with Coomassie blue stain. (A, middle) Purified NPM or NPMdL proteins were incubated overnight with rpL5 and immunoprecipitated (IP) with NRS or antibodies recognizing NPM or rpL5. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with NPM and rpL5 antibodies. (A, right) Purified NPM or rpL5 proteins were incubated overnight with recombinant p27 and immunoprecipitated with NRS or antibodies recognizing NPM, rpL5, or p27. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with NPM, rpL5, and p27 antibodies. α , anti. (B) Proteins from HeLa cell lysates were immunoprecipitated with NRS, rpL5 antibody, or NPM antibodies. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with NPM and rpL5 antibodies. Alternatively, HeLa lysates were pretreated for 1 h with RNase A prior to immunoprecipitation as described above. (C) HeLa lysates were subjected to serial immunoprecipitation with NPM antibodies (lanes 1° to 4°). Precipitated proteins and proteins in the final supernatant (unbound) were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies recognizing NPM and rpL5.

some once it reaches the cytosol. They also indicate that NPM also associates with the 40S subunit, which is devoid of rpL5 (Fig. 6) (30).

Transduction of HeLa cells with His-NPMdL resulted in a dramatic redistribution of rpL5 in cytosolic ribosomes; rpL5 was maintained in the 60S subunits but severely reduced in 80S ribosomes (Fig. 6, middle left panels). Given these findings, we cannot rule out the possibility that rpL5 proteins are still capable of some NPM-independent shuttling. However, it is more likely that rpL5 association with cytosolic ribosomes in the presence of NPMdL is a result of preexisting, stable cytosolic rpL5 complexes. This notion is further substantiated by treatment of HeLa cells with LMB. LMB treatment yielded results that were consistent with NPMdL overexpression (Fig. 6, lower panel). Both NPM and rpL5 proteins were found in the cytosol of LMB-treated cells (at reduced levels), even though the nuclear export of both proteins is LMB sensitive. This finding suggests that some preexisting cytosolic NPM and rpL5 ribosome complexes are fairly stable (~ 24 h) and that, if either protein utilizes CRM1-independent export, it is minimal.

NPM is required for rpL5 nuclear export. Having demonstrated a reduction of rpL5 associated with cytosolic ribosome subunits in the absence of NPM nuclear export signals, we next

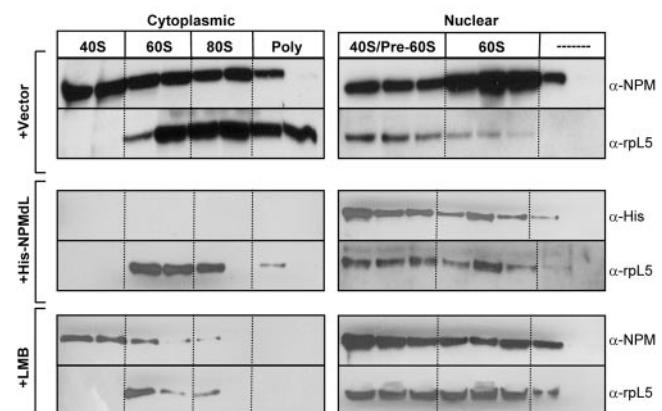


FIG. 6. NPM and rpL5 colocalize with nuclear and cytosolic ribosome subunits. HeLa cells transduced with vector (top panels) or His-NPMdL (middle panels) or treated with LMB (bottom panels) were divided into cytosolic and nuclear fractions and subjected to sucrose gradient centrifugation. Absorbance was monitored at 254 nm, and fractions containing 40S, 60S, 80S, and polysome units were collected. Proteins from each fraction were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies recognizing NPM, the His epitope, and rpL5. α , anti.

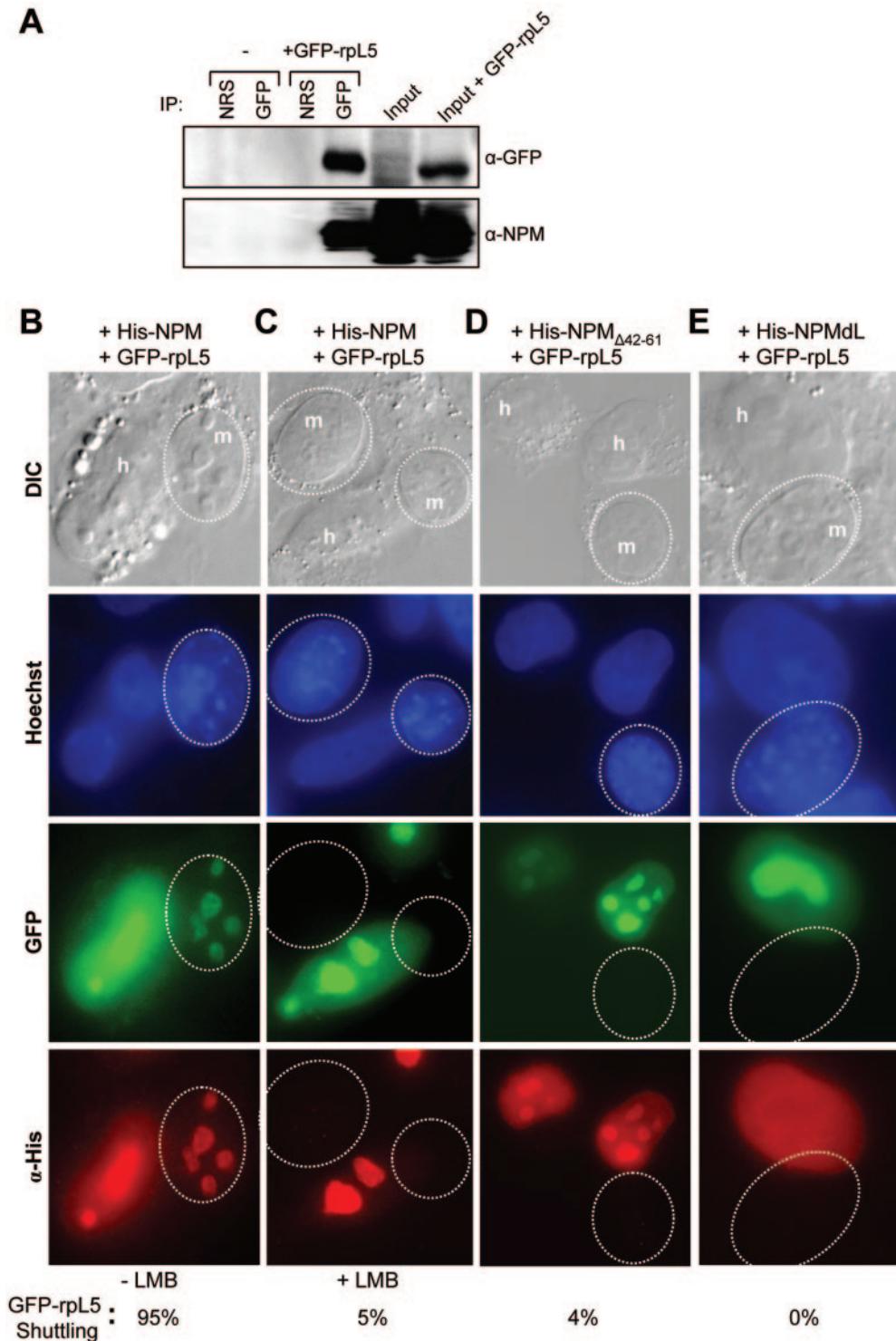


FIG. 7. NPM nuclear export signals are required for the efficient export of GFP-rpL5. (A) HeLa cells either untransfected or transfected with GFP-tagged L5 for 48 h were harvested and lysed. Proteins were immunoprecipitated (IP) with NRS or a rabbit GFP antibody. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with GFP and NPM antibodies. α , anti; DIC, differential interference contrast. Loading inputs are indicated. (B to E) NIH 3T3 cells were seeded onto HeLa cells that had been transfected with GFP-rpL5 in combination with (B and C) His-NPM, (D) His-NPM_{Δ42-61}, and (E) His-NPMdL. Additionally, HeLa cells in panel C were treated with LMB for 18 h prior to fusion. Heterokaryon assays were performed with NPM and GFP-rpL5 proteins being visualized with antibodies against His (red) and naturally emitting GFP spectra (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed in three independent experiments. The percentages of heterokaryons exhibiting GFP-rpL5 shuttling are given.

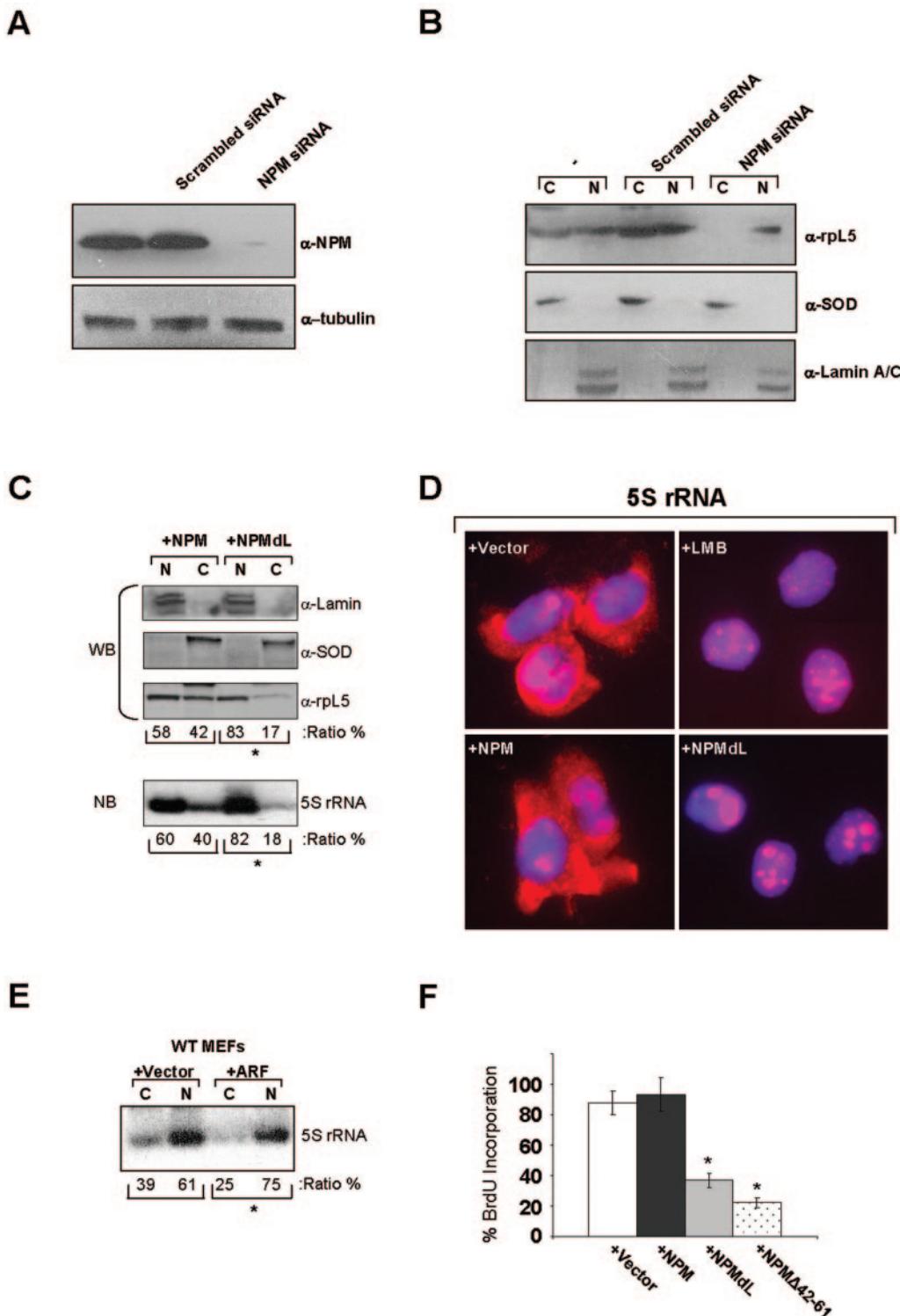


FIG. 8. NPM is essential for rpL5 nuclear export. (A) HeLa cells (−) or cells transduced with siRNAs encoding either scrambled control or NPM-specific sequences were harvested 72 h posttransduction for Western blot analysis. Proteins separated by SDS-PAGE were transferred to PVDF membranes and immunoblotted with antibodies recognizing NPM and γ-tubulin. α, anti. (B) HeLa cells (−) or cells transduced with siRNAs encoding either scrambled control or NPM-specific sequences were harvested 72 h posttransduction for cellular fractionation. Proteins from nuclear (N) and cytosolic (C) fractions were analyzed by SDS-PAGE and immunoblotted with antibodies recognizing rpL5, SOD (cytoplasm control), and lamin A/C (nuclear control). (C) HeLa cells were transfected with His-NPM or His-NPMdL, and 24 h later equal numbers of cells were subjected to fractionation into cytoplasmic (C) and nuclear (N) extracts. L5 protein was detected by Western blot analysis (top panels; WB). Lamin A/C and SOD are shown as nuclear and cytosolic fractionation controls, respectively (top panels; WB). 5S rRNA was detected by Northern blot analysis of total RNA extracted from the nuclear and cytosolic fractions (bottom panel; NB). The ratios of nuclear to cytosolic

examined the direct influence of NPM shuttling mutants on rpL5 nuclear export using a previously characterized GFP-tagged rpL5 protein (41). To confirm that GFP-rpL5 retained the NPM-binding properties of the endogenous rpL5 protein, we transiently overexpressed GFP-rpL5 in HeLa cells and performed Western blot analysis of GFP-immunoprecipitated complexes. As shown in Fig. 7A, precipitated GFP-rpL5 complexes contained endogenous NPM, confirming that the GFP moiety does not adversely affect the formation of NPM-rpL5 complexes in vivo. GFP-rpL5 and His-NPM readily migrated from human nucleoli to mouse nucleoli, as visualized in interspecies heterokaryons (Fig. 7B). However, in the presence of LMB, both GFP-rpL5 and His-NPM failed to shuttle (95% inhibition; Fig. 7C). Introduction of NPM shuttling mutant NPM_{Δ42–61} or NPMdL inhibited GFP-rpL5 shuttling into mouse nucleoli, restricting its expression to human nuclei (Fig. 7D and E; 96% and 100% inhibition, respectively), establishing that NPM nuclear export is required for the export of rpL5. To more definitively show that NPM is required for rpL5 nuclear export, we knocked down NPM expression in HeLa cells (Fig. 8A). Cells lacking NPM protein expression failed to accumulate rpL5 in the cytoplasm, while cells transduced with scrambled small interfering RNA (siRNA) as a control exhibited an equal distribution of rpL5 between the nucleus and cytoplasm (Fig. 8B). These data underscore the necessity of NPM proteins for the efficient transport of rpL5 out of the nucleus and into the cytoplasm.

Ribosomal protein L5 is known to bind specifically to the mature 5S rRNA and aid in its nucleocytoplasmic transport (31, 37, 47). We hypothesized that NPM export, through its influence on rpL5, is the critical determinant for 5S rRNA nuclear export. To test this hypothesis, we performed Northern blot analysis of similar cellular fractions in the presence and absence of NPM shuttling. Indeed, in the presence of dominant negative NPM shuttling mutants, 5S rRNA failed to accumulate in the cytosol and instead was retained in the nucleus in a ratio similar to that for rpL5 (Fig. 8C). In addition to our fractionation studies, we performed RNA FISH to visualize the localization of steady-state levels of 5S rRNA. As shown in Fig. 8D, 5S rRNA was distributed throughout the nucleoli/nuclei and cytoplasm of HeLa cells transduced with empty vector as well as with wild-type NPM. Consistent with our fractionation data, inhibition of NPM and rpL5 nuclear export with LMB or NPMdL resulted in a severe attenuation of 5S rRNA export to the cytosol (Fig. 8D).

To further expand on this theme, we transduced wild-type MEFs with ARF, a known inhibitor of NPM nuclear export (7). Again, in the presence of the ARF tumor suppressor, 5S

rRNA failed to transit to the cytosol and instead was retained in the nucleoplasm (Fig. 8E). These data imply that ARF and NPM mutants defective in shuttling act similarly to prevent rpL5-5S rRNA nuclear export. To determine whether NPM shuttling mutants also prevent cell cycle progression, HeLa cells transduced with NPM expression constructs were labeled with 5-bromodeoxyuridine (BrdU) to measure active DNA synthesis. Similar to ARF's known cell cycle arrest properties (7), cells expressing NPMdL or NPM_{Δ42–61} failed to enter S phase (Fig. 8F). Thus, NPM shuttling activity is not only required for the nuclear export of the rpL5-5S rRNA complex but also necessary for continued cell proliferation.

DISCUSSION

The nucleolus, a highly specialized and structured organelle, has been described as the cell's control center for ribosomal synthesis, maturation, and assembly, with a host of proteins, RNAs, and other factors being implicated in these processes (8). Recently, numerous proteins (cdc14, NPM, cyclin E, Mybbp1a, telomerase reverse transcriptase, and others) have been shown to continuously shuttle from the nucleolus to various subcellular compartments in a regulated manner, providing evidence that the nucleolus is a dynamic site of multiple cellular events (4, 7, 21, 22, 52).

One such protein, NPM/B23, has been linked to a variety of important cellular processes, both in and out of the nucleolus, including ribosome processing, molecular chaperoning, maintenance of genomic integrity, centrosome duplication, and transcriptional regulation (9, 10, 13, 16, 23, 35). Initially, NPM which was imported into the nucleolus from the cytoplasm was presumed to move about the various compartments of the nucleus (6), a feature shared by many critical cell cycle regulators. This shuttling of proteins between the nucleus and cytoplasm is now recognized as a key mechanism for ensuring proper cell cycle progression (39, 43). In previous reports, we and others identified NPM as a novel p53-independent target of the ARF tumor suppressor protein (5, 7, 19). We have since shown that, in response to hyperproliferative signals, nucleolar ARF directly binds NPM, effectively inhibiting NPM's nucleocytoplasmic shuttling. Here, we have further explored the mechanism and significance of NPM intracellular trafficking. First, we have described the CRM1-dependent nuclear export of NPM, identifying the two leucine residues (42 and 44) that are critical to this process. In addition, we have shown that alteration of the NPM NES resulted in the failure of wild-type NPM to be exported out of the nucleolus, providing evidence that these mutations function in a dominant-negative fashion,

accumulation of rpL5 and 5S rRNA are given as percentages of totals (*, $P > 0.001$). (D) HeLa cells were transfected with vector, His-NPM, or His-NPMdL, and cells were plated on glass coverslips. Twenty-four hours later, cells were subjected to RNA FISH with a TRITC-labeled 5S rRNA probe. Nuclei were stained with DAPI. Untransfected HeLa cells were treated with LMB for 18 h prior to RNA FISH analysis. Results of 5S rRNA localization are each representative of three independent experiments. (E) Wild-type (WT) MEFs were infected with control retroviruses or those encoding ARF, and 48 h later equal numbers of cells were subjected to fractionation into cytoplasmic (C) and nuclear (N) extracts. 5S rRNA was detected by Northern blot analysis of total RNA extracted from the nuclear and cytosolic fractions (*, $P > 0.005$). (F) HeLa cells were transfected with vector, His-NPM, His-NPMdL, or His-NPM_{Δ42–61} and plated on glass coverslips. Cells were incubated with BrdU 72 h posttransfection and fixed 20 h later. Fixed cells were stained with antibodies recognizing BrdU and His epitopes and visualized by immunofluorescence using fluorescein isothiocyanate- and TRITC-labeled secondary antibodies, respectively. Cells (100) were counted for each condition in triplicate. Standard deviations are reported as error bars (*, $P > 0.005$).

through the formation of NPM-NPMdL heteromultimers. Thus, NPMdL mimics the effects of ARF induction by directly impeding the nucleocytoplasmic shuttling of NPM through direct interaction, further demonstrating that NPM must exit the nucleolus/nucleus to maintain and promote cell growth.

We have previously proposed that targets of nucleolar sequestration might in fact “ride the ribosome” from the nucleolus to the cytoplasm to engage in growth-promoting events (45). In agreement with this hypothesis, our findings reveal a direct interaction between NPM and rpL5, providing the first physical link between NPM and ribosomal subunits. Much of the field’s focus has been on the putative role of rpL5 in delivering 5S rRNA to the nucleolus, following the initial transcription of 5S rRNA by RNA polymerase III in the nucleoplasm (31, 37, 47). However, it is also possible that rpL5 is a critical player in the export of the large ribosomal subunit (60S), containing 5S rRNA, from the nucleolus/nucleus to the cytoplasm after its assembly. Clearly, the latter events would render themselves sensitive to NPM regulation, given that NPM provides the necessary export signals and chaperoning capabilities (via rpL5) required to transport components of the ribosome to the cytosol. Indeed, inhibition of NPM nuclear export via deletion or mutation of its NES prevented the trafficking of rpL5, an integral component of the 60S ribosomal subunit. Moreover, reduction of NPM expression through RNA interference completely abolished the cytosolic stores of rpL5, underscoring the absolute requirement for NPM in rpL5 nuclear export. Thus, our initial hypothesis of “riding the ribosome” should be revised to “taking the ribosome for a ride.”

While many components of the ribosome, including rpL5, encode their own NESs, it is clear that a single NES forms a relatively weak interaction with CRM1 (25), suggesting a requirement for additional NESs in the efficient export of complexes. Consequently, proteins like NPM and NMD3 may have evolved to serve this purpose. Additionally, NPM and rpL5 were found, in reduced amounts, in cytosolic 40S and 60S complexes, respectively, after LMB treatment, implying either that these particular protein-ribosome complexes are fairly stable or that minor fractions of NPM and rpL5 utilize CRM1-independent modes of transport from the nucleus. Considering that the predominant function of rpL5 is to bind and mobilize 5S rRNA molecules, it was not surprising that 5S transport was also NPM sensitive, and thus NPM contributes to the efficient nuclear export of rpL5-5S rRNA complexes. However, NPM was present in 40S, 60S, 80S, and polysomes in the cytoplasm, implying that NPM, free (within the 40S subunit) or bound to rpL5, remains associated with the mature ribosome as it assembles and forms actively translating polysomes in the cytosol. Taken together, these findings open up the possibility that NPM might transmit additional cues (beyond nuclear export) to cytosolic ribosomes during translation, consistent with nucleolus’s proposed role in dictating translation rates (28).

While it has been appreciated for several decades that changes in nucleolar structure are reliable markers of cellular transformation, experiments that provide a direct link between nucleolar dysfunction and tumorigenesis remain to be conducted. In fact, the nucleolus has largely been dismissed as a static organelle, having little to no impact on the overall well-being of the cell. However, this “nucleolar stigma” recently has been challenged with the discovery that tumor suppressors,

such as p53 and ARF, play a direct role in regulating nucleolar processes (5, 7, 42, 49). Interestingly, rpL5 is also a binding partner of Mdm2 and p53 (12, 17, 29), suggesting that rpL5 may provide an intriguing mechanistic link between ARF and ARF-binding partners. Clearly, through its interaction with NPM, ARF is capable of inhibiting nuclear export of rpL5-5S rRNA complexes. Inhibition of NPM-directed rpL5-5S nuclear export by ARF or NPM mutants defective in shuttling results in cell cycle arrest, demonstrating the importance of rpL5-5S export in maintaining cell proliferation. Moreover, NPM itself is a unique player in both the p53 and ARF responses (10, 11), providing us with a glimpse of how this network of protein interactions may inevitably become sensitive to oncogenic and tumor-suppressive signals in determining tumorigenic cell fates.

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Therapeutic Targets in the ARF Tumor Suppressor Pathway

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ABSTRACT

One of the outstanding fundamental questions in cancer cell biology concerns how cells coordinate cellular growth (or macromolecular synthesis) with cell cycle progression and mitosis. Intuitively, rapidly dividing cells must have some control over these processes; otherwise cells would continue to shrink in volume with every passing cycle, similar to the cytoreductive divisions seen in the very early stages of embryogenesis. The problem is easily solved in unicellular organisms, such as yeast, as their growth rates are entirely dependent on nutrient availability. Multicellular organisms such as mammals, however, must have acquired additional levels of control, as nutrient availability is seldom an issue and the organism has a prodigious capacity to store necessary metabolites in the form of glycogen, lipids, and protein. Furthermore, the specific needs and specialized architecture of tissues must constrain growth for growth's sake; if not, the necessary function of the organ could be lost. While certainly a myriad of mechanisms for preventing this exist via initiating cell death (e.g. apoptosis, autophagy, necrosis), these all depend on some external cue, such as death signals, hypoxia, lack of nutrients or survival signals. However there must also be some cell autonomous method for surveying against inappropriate growth signals (such as oncogenic stress) that occur in a stochastic fashion, possibly as a result of random mutations. The ARF tumor suppressor seems to fulfill that role, as its expression is near undetectable in normal tissues, yet is potently induced by oncogenic stress (such as overexpression of oncogenic Ras or myc). As a result of induced expression of ARF, the tumor suppressor protein p53 is stabilized and promotes cell cycle arrest. Mutations or epigenetic alterations of the *INK4a/Arf* locus are second only to p53 mutations in cancer cells, and in some cancers, alterations in both *Arf* and *p53* are observed, suggesting that these two tumor suppressors act coordinately to prevent unwarranted cell growth and proliferation. The

aim of this review is to characterize the current knowledge in the field about both p53-dependent and -independent functions of ARF as well as to summarize the present models for how ARF might control rates of cell proliferation and/or macromolecular synthesis. We will discuss potential therapeutic targets in the ARF pathway, and some preliminary attempts at enhancing or restoring the activity of this important tumor suppressor.

INTRODUCTION

Since its discovery as a product of the alternate reading frame of the mouse *INK4a/Arf* locus [1], the ARF tumor suppressor has been identified as a key sensor of hyperproliferative signals such as those emanating from the Ras and Myc oncoproteins [2-4]. p16^{INK4a} and ARF are transcribed from separate and unique first exons (over 10 kilobases apart) which splice into two shared exons [1] (Figure 1). While INK4a and ARF share considerable homology at the DNA level (nearly 70%), the translated proteins are completely distinct from one another. This is due to the unprecedented splicing utilized by ARF which causes a frame shift (alternate reading frame) in the coding region of exon two (and thus providing the ARF moniker). The *INK4a/Arf* locus is frequently targeted for loss of function in diverse human cancers and both p16^{INK4a} and ARF function as tumor suppressors despite a lack of sequence similarity. ARF is a highly basic (predicted pI=11), insoluble protein which exhibits little structure apart from a pair of alpha helices at its amino terminus [5]. Both mouse and human ARF have been widely studied in the decade since their discovery. Although they differ in size (mouse ARF is 19 kDa and human ARF is 14 kDa) and exhibit only 49% sequence identity, the functions of the ARF proteins appear to be conserved in mice and men. ARF is a *bona fide* tumor suppressor. Ectopic ARF is capable of arresting immortal rodent cell lines as well as transformed human cells [6, 7],

a classic and requisite property of tumor suppressors. The ability of ARF to inhibit cell cycle progression in numerous cell types, suggested that ARF had powerful growth-inhibitory functions in the cell and prompted many researchers to study the *in vivo* ability of ARF to prevent tumorigenesis.

LOSS OF ARF IN CANCER

Animal studies have been very valuable in elucidating the function of murine p19^{ARF} as a tumor suppressor. *Arf*-null mice, generated by specifically targeting exon 1 β , exhibit spontaneous tumor formation as early as 8 weeks of age [3]. Sarcomas and lymphomas are the most common tumors observed in *Arf*-deficient mice. Tumor development is also accelerated in newborn *Arf*-null mice treated with carcinogens when compared to wild-type mice [3, 8], demonstrating that ARF protects cells against aberrant cell growth and proliferation caused by increased mutagenesis. Another interesting facet of ARF biology is the observed immortal phenotype of cultured *Arf*-null mouse endothelial fibroblasts (MEFs). Unlike its wild-type counterparts which senesce after 10-15 passages *in vitro*, *Arf*-deficient MEFs are capable of growing *infinitum* in culture [3]. Moreover, immortal *Arf*-null MEFs are susceptible to transformation by oncogenic Ras alone, indicating that loss of *Arf* can be substituted for Myc overexpression in classic cooperating transformation assays with Ras [3]. This finding was further refined through experiments that showed the acute loss of *Arf* as a major event in Myc-induced cellular immortalization *in vivo* [9].

Consistent with initial findings in mice, frequent mutation or deletion of the *INK4a/Arf* in numerous human cancers was discovered. It is difficult, however, to assess the relative importance of p16^{INK4a} and ARF individually since mutation or deletion at the *INK4a/Arf* locus

frequently affects both proteins. Mutation of exon 1 β , which would specifically affect only ARF, is a relatively rare event. However, a germline deletion of a region containing exon 1 β of p14^{ARF} but leaving the *INK4a* gene intact was identified in a family prone to melanoma and neural system tumor development [10]. An exon 1 β mutation that altered the growth-inhibitory properties and intracellular localization of human p14^{ARF} was observed and characterized in a melanoma patient [11]. Building on these early reports, ARF haploinsufficiency due to a germline mutation in exon 1 β was observed in a family of three individuals with melanoma or breast cancer. However, somatic changes at the *INK4a/Arf* locus discovered in one of the melanoma samples resulted in inactivation of both p14^{ARF} and p16^{INK4a} [12]. Recently, a germline deletion of exon 1 β was discovered in two patients from a family predisposed to malignant melanoma. A heterozygous germline missense mutation in exon 1 β was also found in another individual with melanoma [13]. More commonly, however, exon 2 is the site of mutation, affecting either p16^{INK4a}, ARF, or both proteins. Some of these exon 2 mutations alter ARF localization and affect its regulation of downstream target proteins [14-16]. Silencing of the *Arf* gene promoter through hypermethylation is frequently observed in low-grade diffuse astrocytomas [17], oligodendroglial tumors [18, 19], ependymal tumors [19, 20], kidney cancer [21], hepatocellular carcinoma [22], colorectal cancer [23, 24] and oral squamous cell carcinomas [25]. Simultaneous methylation of both *Arf* and *INK4a* is also a common occurrence in samples from the accelerated phase of chronic myeloid leukemia (CML) [26]. In one study, loss of p14^{ARF} expression was observed in 38/50 glioblastomas, with 29 displaying either homozygous deletion or hypermethylation of *Arf*. While deletion of both p14^{ARF} and p16^{INK4a} was common, *Arf* was specifically deleted in nine of the samples [27], indicating that ARF alone

is often a major target in human tumor progression (for a complete list of ARF-specific alterations in human cancers, see Table 1).

NUCLEOLAR LOCALIZATION

ARF is predominantly localized to the nucleolus [28, 29], a dynamic, membrane-less, subnuclear organelle which controls ribosome biogenesis [30] (Figure 2A). Within the nucleolus, ARF resides in the granular region, which contains maturing ribosomes. During mitosis, the nucleolus disintegrates causing nucleolar proteins to disperse throughout the nucleoplasm [31]. Interestingly, nucleolar dissociation is linked with an increase in p53 [32], suggesting that the nucleolus may be an important structure involved in regulating the p53 pathway. Nucleolar breakdown due to mitosis or stress may allow transient ARF activity in the nucleoplasm [33, 34], however non-nucleolar ARF exhibits decreased stability [35]. Importantly, the last two years have been marked with increased understanding of the role of the nucleolus in sensing both environmental and oncogenic stress within the cell [32, 30].

Interestingly, the amino acid residues responsible for the nucleolar localization of mouse p19^{ARF} and human p14^{ARF} are somewhat different [36]. While the nucleolus is not partitioned from the nucleoplasm by a membrane, entry into this organelle is not thought to be a passive event. Rather, proteins that reside within the nucleolus often contain arginine and lysine rich domains reminiscent of nuclear localization signals that somehow target them to the nucleolus. However, these positively charged tracts are not obligatory for protein nucleolar localization. In fact, many proteins utilize protein-protein and protein-RNA interactions to “hitch a ride” into the nucleolus. Both mouse and human ARF proteins contain arginine-rich sequences (in fact, both proteins are nearly 25% arginine), albeit in different moieties along ARF’s amino acid sequence.

In particular, residues 26-37 are critical for the nucleolar localization murine p19^{ARF} [29] (Figure 2B). In humans, amino acids 2-14 and 82-101 of p14^{ARF} are important for its nucleolar localization [36, 15, 16] (Figure 2B). Of note, deletion of the nucleolar localization signal within either mouse or human ARF results in a loss of ARF's ability to promote cell cycle arrest [16, 37, 29, 36], revealing that the biological function of ARF is intimately tied to its ability to properly localize to the nucleolus.

ACTIVATION OF P53

ARF is most commonly known for its well-characterized activation of the p53 pathway (Figure 1A). The *p53* gene is the most common target of mutations which inactivate protein function or compromise its expression in human cancers. In fact, p53 is disrupted in greater than 50% of all human cancers. In response to cellular stress, p53 is activated to induce cell cycle arrest or trigger apoptosis depending on the setting. These stress cues include DNA damage, nucleotide depletion, viral infection, heat shock, and oncogenic stimuli. The crucial negative regulator of p53 is the E3 ubiquitin ligase, Mdm2 (Hdm2 in humans). Mdm2 binds to p53 and promotes its nuclear export and degradation through post-translational ubiquitin modification [38]. In the absence of *Mdm2*, p53 activity is unchecked, resulting in unrestrained apoptosis in cells and mice [39, 40]. Conversely, coinciding loss of *p53* and *Mdm2* rescues the apoptotic phenotype and mimics the loss of *p53* alone [39-41].

In response to oncogenic signals such as those emanating from Ras and Myc, ARF is up-regulated and accumulates in the nucleolus. ARF interacts with Mdm2, preventing its nucleocytoplasmic shuttling and drawing it into the nucleolus [42, 43, 29]. In this manner, Mdm2 is sequestered by nucleolar ARF molecules. This liberates p53 in the nucleoplasm where

it is free to activate numerous downstream transcriptional regimens. Both Mdm2 and ARF are transcriptional targets of p53, with Mdm2 expression increased and ARF repressed in a negative feedback loop [44]. Moreover, the main consequences of p53 activation, cell cycle arrest or apoptosis, are mediated by p53 target genes such as $p21^{\text{CIP1}}$ and PUMA, respectively. Recent reports have indicated that the tumor suppressive activities of p53 are mediated by oncogenic activation of ARF and not the DNA damage response [45, 46], suggesting that ARF is the key player in relaying cellular cues to the p53 tumor suppressor.

Interestingly, the residues in ARF (both mouse and human) that are critical for binding to Mdm2 also regulate ARF's nucleolar localization and cell cycle arrest [16, 36]. The amino-terminal 37 residues of $p19^{\text{ARF}}$ (contained within exon 1 β) are sufficient for all of its known properties including its binding to Mdm2 and localization to the nucleolus [29, 36]. Mdm2 also contributes to its nucleolar co-localization with ARF through a cryptic nucleolar localization signal which is revealed upon binding to ARF [37]. The notion that nucleolar sequestration of Mdm2 by ARF is necessary for its activation of p53 has been challenged by reports showing that ARF-mediated regulation of p53 can occur independent of Mdm2 nucleolar re-localization, albeit only when ARF mutants are overexpressed [47, 48]. However, nucleolar sequestration of Mdm2 by PML occurs in response to DNA damage [49], suggesting that Mdm2 re-localization to nucleoli, while not absolutely necessary, may be a common feature in different pathways of p53 activation.

MDM2 INHIBITORS

Through its inhibition of Mdm2 in response to oncogenic stimulation, ARF plays a key role in p53 pathway activation. In cells where ARF expression or function is lost through mutation or

deletion, the aberrant activation of oncogenes does not induce a typical p53 response, but rather results in cellular transformation [3, 50]. *Mdm2* gene amplification, which occurs in tumors expressing wild-type p53 [51], is capable of over-riding the suppressive effects of ARF [52]. Thus, Mdm2 represents a promising target for p53-positive tumors. Direct targeting of Mdm2 with pharmacological inhibitors has the potential to increase p53 protein levels and activity. Furthermore, the use of Mdm2 inhibitors would bypass the normal requirement for ARF in p53's response to oncogenic stimuli, making it an effective therapy in tumors lacking functional ARF.

Several attempts have been made to identify molecules that target the p53-inhibitory activities of Mdm2 with a few promising candidates emerging. The nutlins are a class of Mdm2 inhibitors, identified in a synthetic chemical library screen, which occupy the hydrophobic p53-binding pocket of Mdm2. Nutlins inhibit the interaction between p53 and Mdm2 in a dose-dependent manner *in vitro*. In cancer cell lines that retain wild-type p53, nutlins inhibit cell cycle progression and induce p53 expression and subsequent apoptosis. Nutlin-3 inhibited growth of tumor xenografts in nude mice without any reported side effects over a three week treatment regimen [53, 54]. The HLI98 class of Hdm2 inhibitors was identified from a screen for small molecules which inhibited auto-ubiquitination of Hdm2. Dose-dependent inhibition of p53 ubiquitination and an increase in p53 protein levels and transcription were observed with HLI98. Additionally, HLI98 molecules induced apoptosis and inhibited colony formation. Unlike nutlins, HLI98 molecules do not inhibit the interaction of Hdm2 with p53 [55], but rather the E3 ligase activity of Mdm2. However, HLI98 molecules exhibit limited specificity. Thus, further refinement is needed to improve the feasibility of specifically targeting Hdm2 ubiquitin ligase activity.

Problems surrounding the therapeutic use of Mdm2/Hdm2 inhibitors include potential toxicity in normal tissues due to uncontrollable p53 activity. Moreover, successful inhibition of Mdm2 may well lead to stabilization of p53 but may not elicit a therapeutic response due to other possible mutations in downstream components of the p53 signaling pathway. It seems likely that prolonged treatment with Mdm2 inhibitors may elicit unfavorable responses given a recent report demonstrating severe pathologies in *Mdm2*-null mice conditionally expressing p53 [56]. Deletion of *Mdm2* is embryonic lethal in mice expressing wild-type p53, however *p53/Mdm2* double-null mice are viable, indicating that unrestrained p53 activity is fatal during development [39, 40]. To overcome this hurdle, Ringhausen *et. al.* used a previously described *p53* knock-in mouse model, in which p53 expression was induced by tamoxifen [57], in the context of an *Mdm2*-null background [56]. Tamoxifen administration induced apoptosis and atrophy in radiosensitive tissues and tamoxifen-treated mice died within a week [56]. Therefore, despite great interest in the development of Mdm2 inhibitors, unrestrained p53 activity could be as much of a problem as a solution.

P53-INDEPENDENT TARGETS

Mounting evidence suggests that ARF has a second, p53-independent, function [58, 59]. The most convincing data presented to date involved the use of mouse genetics to confirm that p53 and ARF could contribute independently to suppressing tumorigenesis. Mice lacking *p53* or *Arf* are highly tumor-prone with mean latencies for survival of 19 and 32 weeks, respectively [58]. In mice lacking *p53*, T-cell lymphomas predominate (~70%), with the remainder being sarcomas. In contrast, *Arf*-null mice develop far fewer cases of lymphoma (~25%) and primarily develop poorly differentiated sarcomas (~50%), with the remainder appearing as rare carcinomas and

gliomas [8]. Surprisingly, mice deficient for both *p53* and *Arf* showed a wider range of tumor types than animals lacking either gene alone, and many developed multiple primary tumors without affecting the mean latency of survival (~16 weeks) [58]. To date, more than half of the *p53/Arf*-null animals have developed wide-ranging multiple-type tumors strongly demonstrating that ARF has additional p53-independent functions. Cells devoid of both *p53* and *Arf* grow at a faster rate and are more resistant to apoptotic signals than cells lacking only *p53* or *Arf* [9], demonstrating a cooperative effect of *p53* and *Arf* loss on cell proliferation. This also implies that ARF may functionally interact with proteins other than p53 and Mdm2 to prevent cell growth (see below). While *p53*-null mouse embryo fibroblasts are fairly resistant to ARF overexpression, cells deficient for both *p53* and *Mdm2* are sensitive to ARF-induced growth arrest. This indicates that ARF can act as a *bona fide* tumor suppressor independent of p53 and that Mdm2 can antagonize this effect.

Additionally, in mouse eye development, proper hyaloid vascular regression is dependent upon ARF, but not p53. *Arf*-null mice exhibited accumulation of a retrolental mass, lens degeneration, and lens capsule disruption, symptoms characteristic of the human eye disorder persistent hyperplastic primary vitreous [59]. Induction of p53-independent apoptosis by ARF in colon cancer cells occurs via degradation of CtBP [60]. ARF has also been reported to regulate the transcriptional activities of MYC and E2F1 through direct binding to Myc, E2F1 and DP1, respectively. Regulation of these transcription factors by ARF appears to be independent of p53 or Mdm2 [61, 62]. To date, more than ten binding partners for ARF have been discovered, although one must be overly prudent when considering ARF targets given the incredible basic charge of the ARF protein (pI=11) and the high probability of capturing non-specific binding

partners through classic interaction studies. Further studies involving the biological outcomes of these interactions are certainly warranted.

The addition of a small ubiquitin-like SUMO molecule, in a process known as sumoylation, is a post-translational modification that can alter stability and function of the target protein. Recent evidence has shown that ARF promotes the p53-independent sumoylation of numerous proteins, including Mdm2 [63, 64]. Werner's helicase is sumoylated by ARF, resulting in its redistribution from the nucleolus to other sites within the nucleoplasm [65]. Binding of p14^{ARF} to a SUMO-conjugating enzyme facilitates sumoylation of several proteins including Hdm2, E2F-1, and HIF-1 α . Interestingly, point mutations in p14^{ARF} associated with melanoma altered the ability of ARF to promote sumoylation of Hdm2 or E2F-1 [66], implying that the sumoylation activity of ARF may be a critical component of both its p53-dependent and independent tumor suppressive properties. As such, novel compounds aimed at promoting or mimicking sumoylation of ARF targets may provide a unique mechanism for restoring ARF activity to tumor cells lacking functional ARF.

THE ARF-NPM INTERACTION

Some of the most exciting ARF work in recent years involved the independent discovery of NPM as a nucleolar ARF binding partner by several groups [67, 68, 52, 69]. Nucleophosmin (NPM) is implicated in cancer biology, with both oncogenic and tumor suppressive functions attributed to this relatively abundant protein [70, 71]. Nucleophosmin undergoes CRM1-dependent nucleocytoplasmic shuttling and regulates the nuclear export of ribosomal protein L5 in order to promote ribosome nuclear export [72, 52, 73]. In fact, one p53-independent function of ARF is to inhibit the transport of ribosomal RNAs to the cytosol by sequestering NPM in the

nucleolus [73, 52], reiterating the notion that NPM shuttling is a crucial event in cell cycle progression. Mutations that confer additional nuclear export signals onto NPM, such that NPM rapidly shuttles to the cytoplasm, are associated with acute myeloid leukaemia (AML) [74]. Additionally, chromosomal translocations involving NPM are common in hematological malignancies, while NPM overexpression is observed in diverse tumors [71]. The importance of NPM in maintaining growth and proliferation is underscored by the embryonic lethality observed in *Npm1*-null mice [70, 75].

NPM interacts with ARF in an association that has apparent functional consequences for both proteins. NPM maintains the stability and nucleolar localization of ARF [75, 76, 69] and a cytoplasmic NPM mutant associated with AML redistributes ARF to the cytoplasm and reduces its stability [77, 76], suggesting that while ARF can target the function of NPM, ARF itself can be influenced by NPM oncoproteins [77, 76, 78, 71]. While ARF is stabilized by its interaction with NPM, adenoviral expression of ARF decreased NPM protein levels [67], although other studies have shown that overall levels of NPM remain largely unchanged in cells despite large differences in ARF expression [68, 52]. The interaction of ARF with NPM is mediated by the amino terminus of p14^{ARF} or p19^{ARF} proteins [68, 52, 67]. Notably, this is the same region required for the formation of ARF-Mdm2 complexes. Indeed, ARF preferentially binds to Mdm2 under conditions of equal molar Mdm2 and NPM, arguing that p53-independent functions of ARF might be sensitive to Mdm2 inhibition [52]. This would provide an additional mechanism by which targeted therapeutics against Mdm2 might also reinstate p53-independent functions of ARF.

ROLE OF ARF IN RIBOSOME BIOGENESIS

NPM presents itself as a more teleological target of ARF tumor suppression, one that allows nucleolar ARF to interfere with proper ribosome assembly and export. Recent hypotheses place the nucleolus as a relaying center for the interpretation of growth and proliferation signals. In this sense, ribosome biogenesis is a critical step in both the regulation of mRNA translation and cell cycle progression with alterations in nucleolar function resulting in huge gains in protein synthesis and eventually, enhanced cell growth [30, 32]. How ARF might be involved in these dynamic processes has been debated in recent years. Chromatin immunoprecipitation experiments identified p14^{ARF} at the promoter of rDNA loci and further established a functional interaction between ARF and UBF, a potent inducer of rDNA transcription [79]. Additionally, ARF may act as a checkpoint protein in ribosome biogenesis via inhibition of ribosomal RNA processing [80], resulting in fewer mature cytosolic ribosomes. This potential role seems likely given the localization of ARF in the nucleolus and its ability to inactivate NPM, a key player in ribosome biogenesis. This is further supported by the observation that either ARF overexpression or mutation of the NPM nuclear export signal increased nuclear retention of 5S rRNA [73]. One might conclude that ARF could perform all three functions to ensure that ribosome biogenesis was completely inhibited (transcription, processing and export) (Figure 3) during conditions where ARF is hindering the oncogenic signals presented by Ras and Myc. Loss of *Arf* or overexpression of NPM could increase ribosome biogenesis and accelerate tumorigenesis through tremendous gains in protein synthesis. Thus, the involvement of ARF in the regulation of translation provides a unique opportunity and potential blueprint as to how small molecule inhibitors against NPM might be used to target the ribosome synthesis machinery to prevent tumorigenesis originating from nucleolar dysfunction.

SYNTHETIC ARF PEPTIDES

Recently, peptide delivery has begun to show promise as a legitimate therapeutic strategy, with several studies showing beneficial anti-cancer activity of peptides *in vivo*. Injection of a peptide from the von Hippel-Lindau (VHL) tumor suppressor inhibited the growth and invasiveness of renal tumor implants in nude mice [81]. A peptide containing the D-isomer of a p53 C-terminal fragment was able to activate endogenous p53, inhibit tumor growth, and prolong survival of tumor-bearing mice [82]. Shepherdin, a peptide from survivin that inhibits Hsp90, inhibited tumor growth when injected into mice bearing prostate cancer xenografts [83].

Several studies have indicated that all of the known biological functions of ARF are mediated by the N-terminal amino acids 2-14. Deletion of these residues from mouse and human ARF blocks its recruitment of Mdm2 to the nucleolus, impairs its binding to NPM, and prevents its ability inhibit cell growth and proliferation in both *p53* wild-type and *p53/Mdm2*-null cells [58, 36, 16, 68, 52]. ARFΔ2-14 (lacking residues 2-14) is unable to bind to 5.8S rRNA and subsequently unable to inhibit rRNA processing and proliferation of *p53/Mdm2/Arf*-null MEFs [80]. Residues 2-14 ARF are sufficient for binding Mdm2 and NPM [36, 52] and are required for the sumoylation of ARF target proteins [64], suggesting that this short stretch of conserved amino acids (from mice and men) has considerable potential for use in reconstituting ARF function *in vivo*.

Therapeutic delivery of a small ARF peptide, such as ARF (amino acids 2-14) may mimic the growth-inhibitory effects of full-length ARF expression. In cancers where Mdm2 is overexpressed or where ARF expression is lost through mutation, deletion, or hypermethylation of the *Arf* locus, introduction of a synthetic ARF peptide might restore its regulatory effects on Mdm2. Inhibition of Mdm2 by synthetic ARF peptides may restore p53 activity in these tumors

or, in tumors lacking p53, inhibit ribosome biogenesis (through NPM inactivation) and subsequent cell growth. In fact, expression of a p14^{ARF} peptide containing amino acids 1-20 induced p53 expression and prevented its ubiquitination [84], demonstrating the huge potential of this strategy.

It remains to be determined whether intra-tumoral delivery of ARF peptides is feasible. The unusual amino acid sequence and relative lack of structural information about ARF makes it a challenging candidate as a peptide-based therapeutic. Attachment of a Protein Transduction Domain (PTD) may facilitate delivery of an ARF peptide into the cell, but may also alter its localization. A basic PTD, like that of the HIV TAT protein, is less likely to interfere with the nucleolar localization of an ARF peptide. Additionally, isomers of ARF peptides may enhance its stability and potency without affecting its native nucleolar localization. However, specific targeting of ARF is also a concern, as unregulated p53 activity would be toxic to both tumor and normal cells. Proof-of-principle remains to be established regarding the possible efficacy of ARF peptides as therapeutic anti-cancer agents, but ongoing mutagenesis studies of ARF residues 2-14 could reduce the number of critical amino acids required for ARF function. This would essentially provide chemists with the opportunity to mimic short ARF peptides with the goal of generating chemical compounds that would be capable of inhibiting Mdm2 and NPM function in a manner analogous to ARF.

CONCLUDING THOUGHTS

As a nucleolar tumor suppressor, ARF is positioned to sense and regulate growth in the cell (Figure 4). In response to hyper-growth or hyper-proliferative signals, ARF protein levels increase in the nucleolus leading to cell cycle arrest. Current interest in ARF biology for

pharmaceutical companies certainly lies within the selective inhibition of Mdm2 molecules, as numerous compounds are under pre-clinical and clinical investigation for their efficacy in this regards. While these compounds, from a structural standpoint, may not be true ARF mimics, they could be viewed as functional ARF substitutes with their activities serving to potentiate a p53 response in tumor cells. The major drawback to this approach is in its inherent reliance on an intact *p53* gene. However, genetic evidence suggests that p53-independent targets of Hdm2 may also contribute to the oncogenic capabilities of Hdm2 [85, 86]. If this holds true, then Hdm2 inhibitors may have profound effects in tumors regardless of their *p53* status.

Given its nucleolar localization, it is not surprising that ARF can inhibit numerous steps in ribosome biogenesis. In fact, one could argue that this might be ARF's teleological role in the cell: maintaining ribosome homeostasis, although definitive experiments in this regard are currently lacking. While it is easy to envisage the effect that inhibiting ribosome production would have on the growth of tumor cells, the side effects of inhibiting these processes in normal cells might be too great to utilize this approach clinically. However, new trials with known inhibitors of ribosome production could reverse this pessimistic view. Rapamycin and its chemical analogues are currently in various phases of clinical trials based largely on their ability to inhibit protein synthesis signaling pathways mediated by mTOR [87]. Inhibition of mTOR selectively inhibits both CAP-dependent and TOP-dependent translation as well as RNA polymerase I rDNA transcription, effectively stopping ribosome production and protein translation [88, 89]. In fact, in some inherited cancer pre-disposition syndromes normal cells are unaffected by rapamycin while tumor cell growth and proliferation is halted [90], suggesting that tumor cells might be far more sensitive to translation inhibition. Viewed another way, tumor cells might simply require greater protein production in order to maintain their proliferative

capacity, making them super-sensitive to slight reductions in ribosome output. Under these conditions, ARF mimics (either peptides or small molecules) or ribosome production inhibitors (transcription, processing or export) might be potent inhibitors of tumorigenesis, again regardless of p53 status, without inadvertently affecting normal tissues. Thus, restoring ARF function in tumor cells to activate both p53-dependent and -independent pathways, some of which are only beginning to be elucidated, would provide a formidable block to tumor growth.

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Table 1. Loss of ARF in human cancer. Diseases in which ARF alterations occur are listed alphabetically along with the frequency of occurrence. ARF-specificity refers to whether p16^{INK4a} is simultaneously affected by the loss of p14^{ARF}. ND = Not Determined.

Disease	ARF alteration	Occurrence	ARF specificity	References
acute lymphoblastic leukemia	deletion	40% ; 45%	No	[91, 92]
adult acute myelogenous leukemia	deletion	5%	No	[93]
adult T-cell leukemia / lymphoma	methylation	6%	ND	[94]
anal squamous cell carcinoma	methylation	25%	ND	[95]
anaplastic meningioma	1. loss of mRNA expression	71%	20%	[96]
	2. mutation OR deletion	67%	No	[97]
	3. methylation	50%	ND	[98]
anaplastic oligodendrogloma	methylation OR deletion	40%	25%	[99]
angiosarcoma	methylation	26%	40%	[100]
atypical meningioma	1. loss of mRNA expression	17%	No	[96]
	2. deletion OR methylation	6%	No	[97]
	3. methylation	20%	ND	[98]
astrocytomas (low grade)	methylation	10% ; 20%	ND ; 100%	[101, 17]
astrocytomas (high grade)	deletion	21%	No	[102]
Barrett's adenocarcinoma	methylation	20%	Yes	[103]
benign meningioma	1. loss of mRNA expression	44%	67%	[96]
	2. methylation	9%	N/D	[98]
bladder cancer	1. methylation	56% ; 31%	67% ; N/D	[104, 105]
	2. deletion	43% ; 14%	Yes ; No	[106, 107]
bladder cancer (Schistosoma-associated)	methylation	19%	60%	[108]
brain metastases	methylation	33%	ND	[109]

Disease	ARF alteration	Occurrence	ARF specificity	References
breast cancer/ melanoma/ pancreatic cancer	mutation	familial	No	[110]
breast carcinoma	1. methylation 2. deletion OR methylation	24% ; 19% 20%	54% ; 58% ND	[111, 112] [113]
cholangiosarcoma	methylation	25% ; 38%	62% ; ND	[114, 115]
chronic myeloid leukemia	1. methylation 2. mutation 3. methylation AND mutation 4. methylation OR missense mutation	40% 23% 17% 47%	17% 71% 60% 14%	[26]
clear cell sarcoma	deletion or mutation	14%	No	[116]
colon cancer	methylation	33% ; 22% ; 33%	ND ; ND ; ND	[112, 117, 118]
colorectal adenoma	methylation	32% ; 40%	ND ; ND	[119, 120]
colorectal carcinoma	methylation	28% ; 38% ; 51%	52% ; 50% ; 70%	[120, 23, 24]
cutaneous melanoma	deletion	67% ; 46%	9% ; No	[121, 122]
cutaneous squamous cell carcinoma	1. mutation 2. methylation 3. mutation OR methylation	8% 40% 43%	No 75% ND	[123]
EBV-associated gastric carcinoma	methylation	100%	No	[124]
ependymoma	methylation	21% ; 28%	96% ; Yes	[20, 19]
epithelial ovarian cancer	mutation, methylation, OR loss of mRNA expression	22%	40%	[125]
esophageal squamous cell carcinoma	1. deletion 2. methylation 3. mutation	33% ; 14% 15% ; 52% ND ; 19%	Yes ; ND No ; 73% ND ; No	[126, 127]
Ewing sarcoma	1. deletion 2. methylation, deletion, OR mutation	13% 13%	No No	[128, 129]
gall bladder / bile duct carcinomas	methylation	46%	50%	[130]
gastric cancer	methylation	24% ; 10%	Yes ; ND	[131, 132]
gastrointestinal stromal tumors	deletion OR methylation	32%	No	[133]

Disease	ARF alteration	Occurrence	ARF specificity	References
glioblastoma	1. deletion 2. deletion OR methylation	55% 58% ; 67%	No 45% ; ND	[134, 27, 135]
glioma	deletion	41%	No	[136]
head and neck squamous cell carcinoma	1. methylation	19%; 16%	85% ; ND	[137, 138] [139] [138]
	2. mutation	35%	6%	
	3. methylation, mutation, OR deletion	43%	16%	
hepatocellular carcinoma	1. deletion	25%	No	[140]
	2. methylation	42%	ND	[22]
	3. deletion OR mutation	7%	No	[141]
	4. deletion, methylation, OR mutation	20%	No	[142]
hereditary nonpolyposis colorectal cancer	methylation	33%	ND	[143]
histiocytic sarcoma	methylation	70%	86%	[144]
intracranial germ cell tumor	deletion OR mutation	71%	No	[145]
kidney tumors	hypermethylation	17% ; 18%	71% ; ND	[21, 146]
malignant mesothelioma	deletion	21%	No	[147]
malignant peripheral nerve sheath tumors	deletion	50% ; 46%	No ; No	[148, 149]
mantle cell lymphoma	deletion	19%	No	[150]
medulloblastoma	1. methylation 2. methylation OR deletion	14% 10%	ND 33%	[151, 152]
melanoma	1. deletion	familial	Yes ; Yes	[13, 10]
	2. mutation	familial	Yes ; Yes ; No ; No	[11, 13, 153, 154]
melanoma/breast cancer	germline mutation	familial	ND	[12]
metastatic cutaneous squamous cell carcinoma	mutation	38%	33%	[155]

Disease	ARF alteration	Occurrence	ARF specificity	References
myxoid / round cell liposarcoma	1. methylation 2. homozygous deletion 3. mutation	11% 6% 21%	ND ND ND	[156]
nasal adenocarcinoma	1. deletion 2. methylation	45% 67%	No No	[157]
neurofibromas and neurofibrosarcomas	methylation	5%	ND	[158]
non-Hodgkins lymphoma	deletion OR mutation	11%	No	[159]
non-small cell lung cancer	1. methylation 2. deletion	8% ; 8% ; 30% 18%	ND ; ND ; ND ND	[160-162] [163]
oligoastrocytoma	methylation	39%	ND	[101]
oligodendroglial tumors	methylation	44% ; 41%	78% ; variable	[19, 18]
oligodendroglioma	methylation	37% ; 21% ; 69%	ND ; Yes ; ND	[164, 165, 101]
oral carcinoma	deletion	22%	No	[166]
oral squamous cell carcinoma	1. methylation 2. deletion 3. mutation 4. deletion OR methylation	20% 24% ; 30% 9% 53%	30% Yes ; ND No 12%	[167] [168, 169] [170] [25]
osteosarcoma	1. methylation 2. methylation, deletion, OR mutation	47% 9%	93% No	[171] [128]
primary central nervous system lymphoma	1. deletion OR methylation 2. deletion OR mutation	56% ; 48% 90%	20% ; 13% No	[172, 173] [174]
prostate carcinoma	deletion OR methylation	13%	No	[175]
pulmonary squamous cell carcinoma	methylation	27%	69%	[176]
renal cell carcinoma	deletion or methylation	5%	No	[177]
salivary gland carcinoma	1. deletion 2. methylation	8% 19%	67% 57%	[178]
small bowel adenocarcinoma	hypermethylation	9%	ND	[179]
sporadic colorectal cancer	methylation	50%	ND	[143]

Disease	ARF alteration	Occurrence	ARF specificity	References
squamous cell carcinoma	mutation	14% ; 55%	No ; No	[180, 181]
supratentorial primitive neuroectodermal tumor	methylation	50%	ND	[151]
T-cell acute lymphoblastic leukemia	mutation OR deletion	100%	3%	[182]
transitional cell carcinoma	deletion	25%	No	[183]
ulcerative colitis-associated colorectal cancer	methylation	50%	ND	[184]
urothelial cell carcinoma	homozygous deletion	22%	No	[185]
Wilms' tumors	methylation	15%	83%	[186]
xeroderma pigmentosum-associated skin carcinoma	mutation	29%	No	[187]

Figure Legends

Figure 1. *INK4a/Arf* locus and effector pathways. **A.** *INK4a* inhibits the activity of cyclin D- cdk4 holoenzymes to keep Rb hypo-phosphorylated and active. ARF blocks the activity of Mdm2 thereby activating p53 as well as inhibiting NPM shuttling activity to prevent ribosome biogenesis. In addition, ARF attenuates the activity of several other proteins although the biological outcomes of these interactions are still being studied. **B.** The *INK4a/Arf* locus. Using an uniquely conserved arrangement of exons, *INK4a* (Exon 1 α , light green) and *Arf* (Exon 1 β , dark green) splice into common 2nd and 3rd exons but in alternate reading frames to produce to distinctive amino acid sequences and structurally unrelated proteins.

Figure 2. ARF nucleolar localization. **A.** Wild type mouse embryonic fibroblasts show ARF (green) localized to the subnuclear organelle, the nucleolus. Nuclear DNA (blue) and

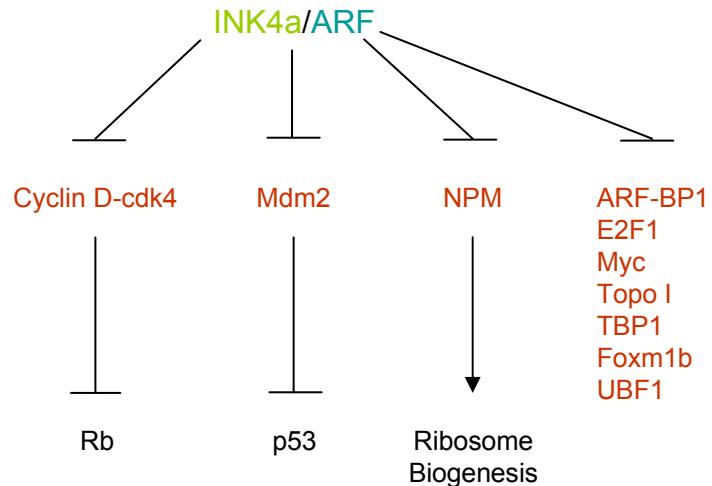
cytoplasmic actin (red) are also shown. **B.** Alignment of Exon1 β and Exon 2 is shown for mouse and human ARF with Mdm2 and NPM binding sites (green) and nucleolar localization signals (blue) shown.

Figure 3. ARF and ribosome biogenesis. The processes of ribosome biogenesis from transcription of rDNA loci to translating polysomes with the known steps sensitive to ARF inhibition are shown.

Figure 4. ARF as a therapeutic agent. ARF mimics could be used to combat tumorigenesis through inhibition of cellular growth by arresting ribosome biogenesis or blocking cellular proliferation through activation of p53.

Figure 1.

A.



B.

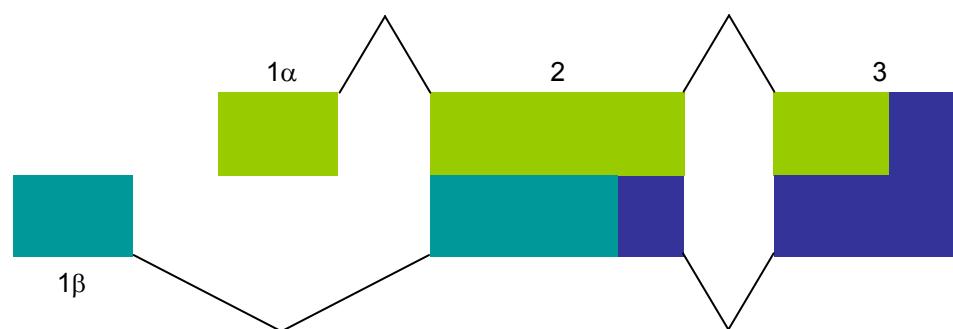


Figure 2.

A.



B.

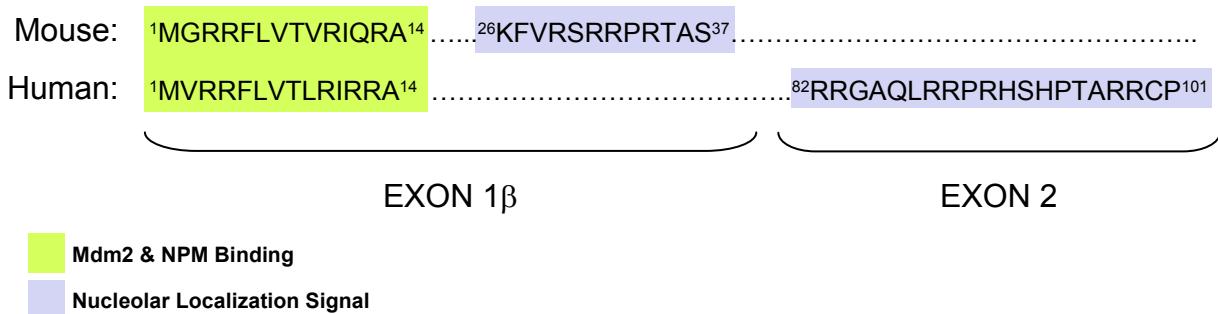


Figure 3.

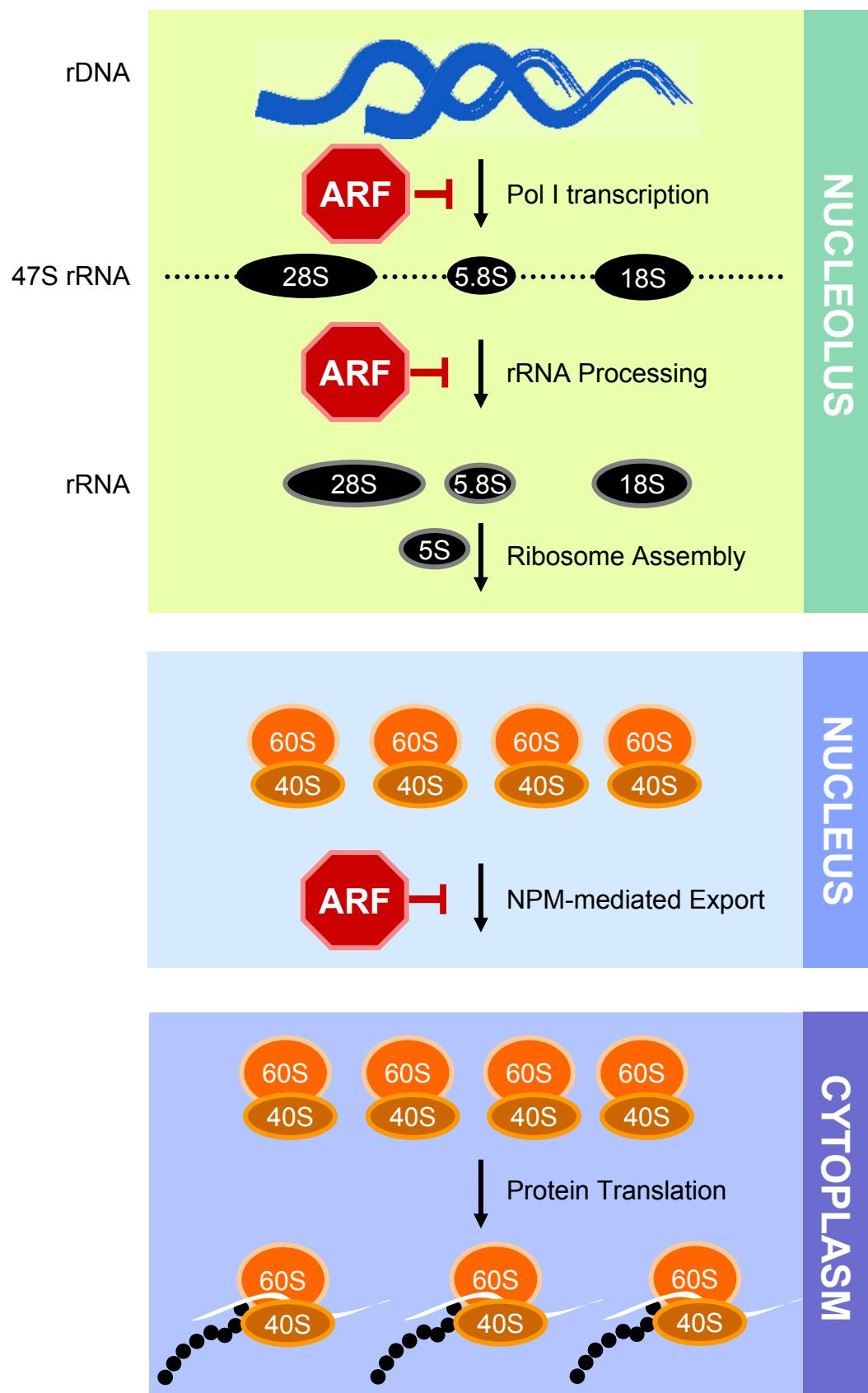
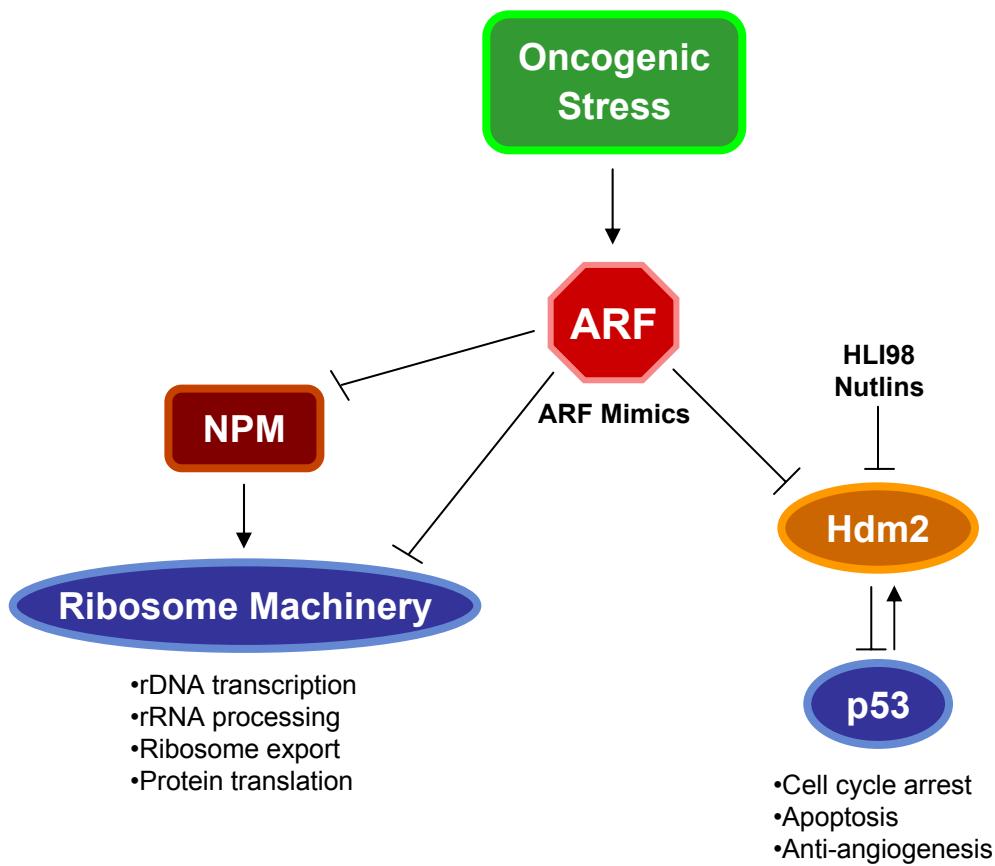


Figure 4.



A Non-tumor Suppressor Role for p19ARF in Maintaining Nucleolar Structure and Function

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ABSTRACT

Cellular growth (macromolecular synthesis) must be coupled to cell proliferation for proper transit through the cell cycle. The factors underscoring cell cycle control have been well studied. However, our knowledge of mechanisms that control cell growth in response to environmental cues is lacking. The nucleolus is at the center of growth sensing; it is the site of ribosome assembly, with nucleolar nucleophosmin (NPM) and p19ARF proteins antagonizing one another to either promote or inhibit growth, respectively. While ARF vigorously responds to hyperproliferative signals to shunt growth, we first noticed that nucleoli from *Arf*^{-/-} MEFs displayed increased nucleolar area, suggesting that ARF might regulate key nucleolar functions in a pre-malignant cell. Ultrastructural analysis of *Arf*^{-/-} nucleoli revealed increased irregularity and larger, more numerous fibrillar centers. In accord with these dysmorphic nucleoli, ribosomal content and total protein synthetic rates were dramatically elevated in the absence of *Arf*. Similar results were obtained using targeted lentiviral RNA interference of *Arf* in wild-type cells, further implicating basal ARF proteins in the regulation of nucleolar structure and function. Finally, *Arf*^{-/-} osteoclasts, post-mitotic cells whose activities are intimately tied to their protein synthesis rates, exhibited enhanced differentiation and resorptive functions, demonstrating a physiological function for ARF in maintaining proper basal protein synthesis *in vivo*. Taken together, these data indicate that disruption of *Arf* greatly impacts ribosomal biogenesis and translational control, providing a significant teleological role for ARF as a monitor of cellular growth independent of its ability to prevent unwarranted cell cycle progression.

INTRODUCTION

Cellular growth (i.e. macromolecular synthesis) is an essential function during the early parts of the cell cycle. In order for cells to make it past the G1 restriction point, sufficient synthesis of DNA licensing factors, cyclins and enzymes of the DNA/RNA replication machinery must occur (). Furthermore, the cell must nearly duplicate its protein content; a failure to do so would result in smaller and smaller daughter cells lacking in the necessary components for continued cell proliferation (e.g. early embryonic division) (). While the mechanisms driving the progression of the cell cycle have been largely worked out, little is still known about the fundamental control of cell growth and how it must be intimately linked to cell cycle progression. Developments in the understanding of how the cell senses environmental nutritional cues has lead to a flurry of research on understanding the fundamental nature of growth control (). With the development of several genetic models in *Drosophila* () and the mouse () that demonstrate aberrations in organ and cell size, molecular pathways involved in these phenomena are now being elucidated. Not surprisingly, several of these pathways converge on the regulation of translation and the synthesis of new ribosomes.

It is estimated that approximately 50% of the cell's energy expenditure is directed toward ribosomal biogenesis (2). The nucleolus, long recognized as a marker for active cellular growth, was first described as the center of ribosomal biogenesis and rDNA transcription in the early 1960's (3, 4). This organelle is composed of three regions on the basis of morphology at the ultrastructural level: the fibrillar centers, the dense fibrillar compartment and the granular compartment. Ribosomal DNA transcription occurs in the junction region between the fibrillar centers and the surrounding dense fibrillar centers, and the resulting rRNA is further processed in

the periphery of the dense fibrillar center. Further post-transcriptional modifications and assembly into subunits occurs in the surrounding granular compartment (5).

While the underlying mechanisms regulating these processes have only begun to be worked out in yeast (), multicellular organisms demand more complex regulatory mechanisms, in that proliferative capacity is not only determined by the relative abundance of nutrients, but also by complicated extracellular signals and growth factors. Indeed, previous studies have demonstrated convergence between the growth and proliferation pathways via regulation of the tumor suppressor genes, Rb and p53 (). Both are known to negatively regulate the activity of PolII () in the transcription of rRNA. In addition, oncogenes such as c-myc also regulate the transcription of rRNA and the mRNAs that encode for ribosomal proteins. ()

The tumor suppressor p19^{ARF} represents yet another attractive candidate for coupling proliferation to growth. Given its nucleolar localization () and potent induction by hyperproliferative signals (), it represents a potential integrator of growth signals coming into the cell. It has been classically associated as a positive regulator of p53 stability through its ability to sequester the E3 ubiquitin ligase for p53, mdm2, in the nucleolus, thereby inactivating it. However, recent data from our () and several other laboratories (), have demonstrated a role for Arf in binding to, and affecting the function of the ribosomal chaperone, nucleophosmin (NPM), independent of its ability to regulate p53. Furthermore, loss of Arf expression or dysregulation through methylation events is observed in over 40% of all cancers, second only to p53 (), and mice lacking both Arf and p53 display an altered tumor spectrum when compared to mice null

for either one alone, suggesting a p53-independent function for Arf as a *bona fide* tumor suppressor.

Given Arf's localization, role in suppressing cellular growth and proliferation, and its nucleolar binding partners, we wished to explore the functional and physiological consequences of disruption of the Arf pathway on growth and ribosomal biogenesis, both *in vitro* and *in vivo*, through both targeted knock-out mice and selective knockdown via lentiviral shRNA delivery. Arf-null mouse embryonic fibroblasts display a significant change in nucleolar morphology and abundance, and have a marked increase in basal protein synthetic levels when compared to wildtype cells. Furthermore, this increase in protein synthesis is in part due to increased ribosomal biogenesis and cytoplasmic content, implying a regulatory role for Arf in this process. . Most surprisingly, though Arf levels are nearly undetectable in low passage MEFs, knockdown of Arf via shRNA constructs exactly mimics the Arf null nucleolar and ribosomal phenotype, implying an important homeostatic role for Arf in wildtype cells. These *in vitro* data are further corroborated by enhancement *in vivo* of osteoclast functionality, a post-mitotic cell dependent on high levels of protein synthesis for proper differentiation. Taken together, these data strongly argue for a moment-to-moment "thermostat-like" role for Arf in controlling ribosomal biogenesis and protein synthetic rates.

MATERIALS AND METHODS

Mice—Arf^{-/-} mice were rederived from TKO heterozygous mice (*Arf*^{+/−}, *Mdm2*^{+/−}, *p53*^{+/−}, a generous gift from Gerald Zambetti, St. Jude's, Memphis, TN) onto a pure C57/BL6 background by several generations of backcrosses to wildtype C57/BL6 mice, followed by breeding to homozygosity. Age-matched wildtype C57/BL6 mice are used as controls where indicated. All mice were maintained in the Washington University School of Medicine barrier facility.

Cell culture, reagents, and antibodies -- Low passage mouse embryonic fibroblasts (MEFs, passage 3-7) were isolated and maintained as described in DMEM supplemented with 10% fetal bovine serum, 10 µg/ml gentamicin, 1x non-essential amino acids, 1 mM sodium pyruvate, and 2 mM glutamine. () Osteoclasts were generated as previously described () from bone marrow-derived macrophages isolated from mice and then subjected to six days of differentiation with g/ml M-CSF and g/ml RANKL. Rabbit anti- p16^{INK4A} (sc-1207) and goat anti- γ -tubulin (sc-7396) were purchased from Santa Cruz. Rat anti-p19^{ARF} (NB 200-169A) was purchased from Novus biologicals. Mouse anti-nucleophosmin (32-5200) was purchased from Zymed.

Plasmid constructs – pLKO-GFP, a lentiviral shRNA expression vector was a generous gift from Dr. Sheila Stewart (Washington University). To obtain the Arf shRNA vector, pLKO-GFP was digested with AgeI/MluI and annealed oligos containing the shRNA target (nucleotides 157-177 of exon 1 β of p19^{ARF}) or a scrambled control were cloned into these sites, and resultant clones verified by sequencing. Oligos are as follows: ArfshRNAF (sense) 5'CCGGGCTCTGGCTTCGTGAACATGCTCGAGCATGTTCACGAAAGCCAGAGCTTT

TA3',ArfshRNAR(antisense)5'CGCGTAAAAAGCTCTGGCTTCGTGAACATGCTCGAGC
ATGTTCACGAAAGCCAGAGC3',scrF(sense)5'CCGGTACGACCTGAACTGCTTAGGACT
CGAGTCTAAGCAGTTCAAGGTCGTATTTTA3',scrR(antisense)5'CGCGTAAAAATACG
ACCTGAACTGCTTAGGACTCGAGTCTAAGCAGTTCAAGGTCGTA-3'. The underlined portion represents the 21 nt hairpin sense and antisense strands.

Cell staining and immunofluorescence – For AgNOR staining and immunofluorescence, MEFs were seeded onto glass coverslips overnight, and fixed and stained the following day. For osteoclast tartrate-resistant alkaline phosphatase staining (TRAP stain), differentiating osteoclast cultures in six-well plates were fixed with _____ and (insert staining procedure).

The AgNOR staining method is modified from the protocol presented by Aubele et al. (). Briefly, cells were fixed in 2% glutaraldehyde for 10 minutes, followed by a postfixation in a 3:1 ethanol:acetic acid solution for 30 minutes at RT. Cells were stained at 37°C for 9 minutes with a 0.33% formic acid/33.3% silver nitrate solution in 0.66% gelatin, followed by a water rinse and mounted on slides with Vectashield (Vector Labs)

Histomorphometry -- Histomorphometric analysis was performed with OSTEOQUANT Nova Prime software () on images captured at 200x by an Optitronics Magnifire camera on a Nikon TE300 microscope. Total number and total area (in μm^2) of AgNORs per nucleus from 100 nuclei were assessed, and statistical significance determined using Student's t-test.

Electron microscopy – Asynchronously growing wildtype and Arf -/- MEFs were trypsinized and fixed with glutaraldehyde in _____ for 10 minutes. Samples were further processed by the

Washington University Department of Cell Biology's electron microscopy core, and pictures of nuclei and nucleoli taken at 3000x and 7000x, respectively.

³⁵S-methionine incorporation assay – 1×10^5 cells were seeded in six-well plates in triplicate, and then starved of methionine and cysteine for 30 minutes. Cells were pulsed with 14.3 μCi of ³⁵S-methionine (Redivue cell labeling mix, Amersham) for various time points, and then immediately washed twice with cold PBS and lysed with 1% Triton X-100 buffer. Total protein was precipitated from lysates with 10% trichloroacetic acid and pelleted at 14,000 rpm. Pellets were subjected to liquid scintillation counting to measure incorporated cpm.

Ribosome fractionation – 2×10^6 cells were treated with 50 $\mu\text{g}/\text{ml}$ cycloheximide for 10 minutes prior to trypsinization and lysis, and fractionation was carried out over a 10-45% sucrose gradient in a Beckman SW41 rotor at 36,000 rpm, as previously described (). Gradients were fractionated and RNA absorbance at 254 nm was continuously monitored to detect ribosomal subunits.

Lentiviral production and infection – 293T (5×10^5 cells) were transfected with one μg of pLKO-GFP containing either scrambled or Arf shRNA cassettes along with the pHRS.2ΔR packaging vector and the pCMV-VSV-G envelope vector using the FuGENE transfection reagent (Roche). Viral supernatants were collected at 30 hours post-transfection, pooled, and stored at -20°C until use. Wildtype MEFs were plated at a density of 8×10^5 cells/100 mm dish, and infected with 5 ml of viral supernatant containing 10 $\mu\text{g}/\text{ml}$ protamine sulfate. Four hours later, the infection was stopped by removing the virus and changing the media. The cells were infected again the

following day, checked for GFP expression, and allowed to express the shRNA construct for 48 hours, after which time they were trypsinized for use in experiments.

Western blot and serial immunoprecipitation—30 µg of RIPA cell extract from MEFs in 1x Laemmli buffer was loaded onto 4-20% precast SDS-PAGE Endurance gels (ISC Biosciences), transferred to PVDF membrane (Millipore) and probed with rat anti-p19^{ARF}, goat anti- γ -tubulin, and rabbit anti-p16^{INK4A} (1:500) **need to add info about other ab's**. Secondary HRP conjugated-anti-rabbit, -goat or -rat (Zymed, 1:10,000) and ECL+ (Amersham) were used to visualize bands. For serial immunoprecipitation, 200 µg of wildtype MEF lysate in Tween-20 containing buffer was immunoprecipitated with Gammabind (Amersham) by a custom-made rabbit NPM antibody (Sigma Genosys) against amino acids x-y. The first three immunoprecipitations were carried out overnight, while the fourth and fifth were done for one hour. The final supernatant was concentrated with Vivaspin columns (Vivascience), and all samples were loaded onto 10% SDS-PAGE gels for immunoblot analysis.

47S rRNA realtime RT-PCR – Measurement of 47S rRNA transcript levels was determined as previously described by Cui and Tseng. Briefly, total RNA was isolated from equal numbers of wildtype and Arf $^{-/-}$ cells using Trizol (Invitrogen), and subjected to reverse transcription with a mouse rRNA-specific primer (5'-CGTGGCATGAACACTTGG-3'). Real-time PCR was performed with iQ SYBR Green supermix (Biorad) according to the manufacturer's protocol, with forward primer 5'-CTGACACGCTGTCCTTCCC-3' and reverse primer 5'-GTGAGCCGAAATAAGGTGGC-3'. Absolute copy number was obtained by comparison to serial dilutions of a known amount of plasmid containing the mouse rDNA repeat.

³H –uridine and –methylmethionine labeling experiments-- Equal numbers of wildtype and *Arf* -/- MEFs were starved in methionine-free media containing 10% dialyzed fetal bovine serum. For uridine labeling, cells were labeled for 30 minutes in media containing 2.5 µCi/ml [³H]-uridine (Amersham), then chased in cold media for the appropriate times, whereas cells were treated with 50 µCi/ml [methyl-³H]-methionine (Amersham) for 30 minutes and chased for the appropriate times in cold methionine-containing (10µM) media in the nuclear/cytoplasmic fractionation experiments. Total RNA was isolated using Trizol reagent (Invitrogen), and loaded onto 1% agarose-formaldehyde gels for the uridine experiments. Cellular fractionation was carried out according to the manufacturer's protocol using the Active Motif Nuclear extraction kit (Active Motif). Total RNA was isolated from the nuclear and cytoplasmic fractions using Trizol, and loaded onto 1% agarose-formaldehyde gels. Following electrophoretic separation, RNA was transferred to Hybond N+ membranes (Amersham), crosslinked, and sprayed with EN³HANCE (Perkin Elmer), and subjected to autoradiography at -70° C.

RESULTS

Loss of p19^{ARF} expression enhances protein synthesis and ribosome biogenesis independent of proliferation

Previous reports have demonstrated that *Arf* -/- cells have a growth advantage relative to wildtype cells () as well as having increased rates of ribosomal rRNA processing; one possible explanation could be enhanced protein synthetic rates secondary to increased ribosomal content. To test this hypothesis, we performed ³⁵S-methionine pulse labeling experiments, whereby we measured the amount of incorporated radioactivity into proteins over a one hour time scale. As shown in **Figure 1A**, *Arf* -/- MEFs had an approximately 12-fold increase in incorporated ³⁵S-

methionine over wildtype cells after 24 hours of pulse time, suggesting that these cells protein synthetic rate was vastly higher. Furthermore, these increased protein synthetic rates were not related to any increase in proliferation rates, as the slope of the growth curve for both wildtype and *Arf* -/- MEFs was virtually identical (**Figure 1B**). To determine if these observed differences were due to increased ribosomal content, we performed sucrose density gradient rate-zonal ultracentrifugation of cytoplasmic lysates from these cells to separate the various ribosomal subunits, followed by fractionation of the gradient and real-time absorbance monitoring at 254 nm to detect the relative amounts of ribosomal rRNAs present in each of the subunit fractions. When compared to wildtype lysates, *Arf* -/- cells had significantly more absorbance in the actively-translating polysome fraction indicating a relative abundance of these ribosomal components (Figure 2B). We therefore postulate that p19^{ARF} must act as a negative regulator of ribosomal biogenesis, export, and/or control of translation as others have previously suggested ().

p19^{ARF} is required for proper nucleolar morphology

Staining methods utilizing reduction of silver on argyrophilic proteins surrounding the nucleolar organizing region (NOR) have been used for decades as a prognostic factor in certain carcinomas, wherein increases of the AgNOR index tend to correlate with poor prognoses (). While much literature has examined this correlation, to date, no studies have been performed on pre-malignant, immortal cell lines which may bear resemblance to hyperplastic or hamartomatous tissue found in several genetically predisposed cancer syndromes (e.g. Tuberous sclerosis, neurofibromatosis, Peutz-Jaeger, Li-Fraumeni, etc.). Given p19^{ARF}'s established role as a nucleolar regulator of mdm2/p53 () and as a sensor of hyperproliferative signals (), we reasoned that it might also play a role in maintaining proper nucleolar morphology. To this end,

we adapted an AgNOR staining protocol from Aubele, et al. () for use on mouse embryonic fibroblasts (MEFs) derived from wildtype and *Arf* -/- C57/BL6 mice. AgNOR staining of *Arf* -/- MEFs demonstrated markedly increased numbers of AgNORs/nucleus and a distinct, irregular shape when compared to the fewer numbers and more rounded, symmetric shape of AgNORs in wildtype counterparts (**Figure 2A**). At the ultrastructural level, we also observed multiple, elongated, irregular nucleoli in *Arf* -/- cells, compared to the round nucleoli of the wildtype cells (**Figure 2B, top panels**). These irregularities were also associated with larger fibrillar centers, the sites of rDNA transcription (**Figure 2B, bottom panel arrows**). We quantitated the total nucleolar area per nucleus (a common pathological definition of the AgNOR index (), **Figure 2C**), and observed a 20% increase in *Arf* -/- cells ($31.6 \mu\text{m}^2$ vs. $26.4 \mu\text{m}^2$, n=100, p<<0.001). A significant increase in AgNOR number/nucleus was also observed (5.78 vs. 3.49, n=100, p<<0.001). Taken together, these data suggest a role for p19^{ARF} in maintaining proper nucleolar structure.

Acute knockdown of p19^{ARF} exactly mimics the phenotype of *Arf* -/- cells

Since Arf's effects as a sensor of hyperproliferative signals and its concomitant induction of a p53-dependent cell-cycle arrest have been well established (), it has been assumed that Arf plays little, if any, function in the normal day-to-day regulation of cellular homeostasis. Indeed, Arf levels in asynchronously growing wildtype MEFs are virtually undetectable by Western analysis (). Given our findings with *Arf* -/- cells, however, we wished to reexamine this question by acutely knocking down the level of Arf in wildtype cells using lentiviral constructs containing an shRNA duplex that recognizes bases 157 through 177 in the ARF-specific exon 1 β of the *Cdk2na* locus. To verify the specificity of this construct, we infected wildtype MEFs with

lentivirus containing either shRNA specific to Arf, or a scrambled irrelevant control sequence. As shown in the Western analysis (**Figure 3A**), infection with virus containing the Arf shRNA sequence produced a robust knockdown of the level of Arf without also decreasing p16^{INK4A} levels, or affecting other nucleolar proteins such as NPM and ribosomal protein L5. We then proceeded to evaluate our knockdown cells via AgNOR staining, ³⁵S-methionine incorporation, and cytoplasmic ribosome content (**Figure 3B-E**). We again observed a marked morphological change in AgNOR staining akin to that seen in the *Arf* -/- MEFs, with significant increase in both number of AgNORs/nucleus (6.6 vs. 3.3, n=100, p<<0.001) and total AgNOR area/nucleus (49.8 μm^2 vs. 36.8 μm^2 , n=100, p<<0.001). As with the *Arf* -/- MEFs, the knockdown cells also displayed a significant enhancement of the polysomal subunit peaks, and a 10-fold enhancement in ³⁵S-methionine incorporation over 24 hours. Taken together, these results strongly implicate Arf in a baseline regulatory role over protein synthesis and ribosomal biogenesis.

The effects of p^{19ARF} on ribosome biogenesis are not dependent on the cell cycle

To demonstrate a physiological function for Arf's baseline regulation of ribosome biogenesis and protein synthesis, we focused on the bone-reabsorbing cells, the osteoclast, as a model of a cell with high protein synthesis demands. Osteoclasts are formed by fusion of hematopoietically-derived macrophages into multinucleated giant cells with a specialized ruffled-border containing thousands of vacuolar H⁺-ATPases. The osteoclast makes a tight seal against the area of bone reabsorption, in doing so, this allows the specialized ruffled membrane to secrete collagenases and dramatically lower the pH through the activity of the proton pumps (). As such, the osteoclast has a high demand for protein synthesis, since the H⁺-ATPases are specific to the

mature osteoclast, and are not found in macrophage precursors. () Furthermore, since the mature osteoclast is a post-mitotic cell, it affords an excellent opportunity to examine Arf's effects on protein and ribosome metabolism independent of proliferation.

We first examined the osteoclastogenic potential of *Arf* -/- bone marrow-derived macrophages by inducing differentiation with M-CSF and RANK ligand. After three days of stimulation with RANK ligand, cells were fixed and stained with Tartrate Resistant Acid Phosphatase (TRAP) substrate, an osteoclast-specific stain (**Figure 4A**). We observed an increased number of mature osteoclasts derived from *Arf* -/- precursors (arrows), with few, if any, mature osteoclasts in the wildtype control wells. We counted TRAP-positive cells with greater than 5 nuclei as a way to differentiate maturing osteoclasts versus immature precursors, and observed a highly significant increase in the *Arf* -/- wells (**Figure 4B**, 149 vs. 91 per well, n=5, p=0.01).

To determine if the differences seen in osteoclastogenesis translated into increased per cell functionality, we compared the TRAP activity (a marker of osteoclast function) of equal numbers of TRAP-positive cells as determined above. We incubated cell lysates from day 4 post-RANKL addition (for wild type) and day 3 post-RANKL addition (for *Arf* -/-), where we observed approximately equal numbers of TRAP-positive cells, with *p*-nitrophenyl phosphate, a colorimetric substrate for TRAP, and measured absorbance at 405 nm. As depicted in **Figure 4C**, we observed a two-fold increase in TRAP activity (p<0.01). This increase could not be accounted for by increased numbers of TRAP-positive preosteoclasts, as BrdU labeling of day 2 preosteoclasts demonstrated no significant difference in the proliferation rates of wild type and *Arf* -/- osteoclast precursors (**Figure 4D**). We therefore are confident that the differences observed in TRAP activity are representative of the enhanced protein synthetic rates we observed in MEFs, and are not just a function of increased cellular proliferation.

Loss of p19^{ARF} increases rRNA transcription, ribosomal biogenesis and export

Given our previous results that loss of Arf leads to enhanced ribosomal biogenesis and protein synthesis, we wished to examine potential mechanisms by which this might occur. Previous reports () have demonstrated a role for Arf in rRNA processing, and our lab has previously demonstrated Arf's inhibitory ability on the shuttling of the nucleolar protein, NPM (). We have further shown that NPM is a necessary cofactor for the shuttling of both the 5S rRNA and the ribosomal protein L5 (), and that overexpression of a shuttling mutant leads to a gross reduction in the cytoplasmic content of both the 28S and 18S rRNAs, as well as all cytoplasmic ribosomal subunits. Taken together, this strongly implicates a role for Arf in regulating ribosomal rRNA shuttling through NPM. As seen in **Figure 5A**, loss of Arf has no impact on the levels of either NPM or L5, suggesting that Arf's effect in this pathway is not due to altered synthesis and/or destruction of these proteins. Furthermore, serial immunodepletion of NPM reveals two distinct pools of Arf; one which is effectively in a complex with NPM, and second NPM-independent pool (**Figure 5B**). This implies that Arf's effects in ribosomal biogenesis may not be relegated to just NPM-dependent processes.

To determine if loss of Arf results in decreased rRNA synthesis and/or nuclear-cytoplasmic shuttling, we performed a number of rRNA labeling experiments followed by a pulse-chase analysis. Loss of Arf resulted in a four-fold increase in steady-state primary 47S rRNA transcript in asynchronously dividing MEFs, suggesting that the rate of rRNA transcription is indeed increased (**Figure 5C**). To further underscore this finding, newly synthesized rRNA as determined by [³H]-uridine pulse-chase analysis is markedly increased in the *Arf* *-/-* cells after 120 minutes of chase in cold media (**Figure 5D**). Additionally, nuclear-cytoplasmic shuttling is

also affected by loss of Arf, as a greater proportion of newly synthesized and processed rRNAs are retained in the nuclei of wildtype MEFs following 240 minutes of chase when compared to *Arf* -/- cells (**Figure 5E**, compare lanes 3&4 with 7&8). Finally, this effect is observable via liquid scintillation counting even as early as 30 minutes following the metabolic labeling pulse (**Figure 5F**), indicating that moment-to-moment alterations in Arf levels may produce robust and rapid responses with regards to cytoplasmic ribosomal content.

DISCUSSION

Coupling cellular proliferation to increased macromolecular synthesis has always been a key unanswered central question in biology. While intuitively obvious that this must be the case, the important players have yet to be identified. Recently, several candidates have emerged in yeast, including cyclins () and factors having a role in the control of ribosome biogenesis (). However, the search for such factors in multicellular organisms has largely been met with frustration.

Arf represents a potential first step in that understanding in that it is uniquely positioned at the nexus of both growth and proliferation pathways. While long appreciated for its ability to positively regulate p53 levels in the cell () and serve as a sensor of hyperproliferative signals (), the relatively low abundance of the protein in interphase cells implied that Arf functioned as a cellular checkpoint on growth, thereby requiring a certain threshold level to elicit a response. Here we provide data that questions that idea and demonstrates that the physiological low level of Arf has a *positive* regulatory role in nucleolar function and ribosome biogenesis. Indeed, as soon as 96 hours following Arf knockdown by lentiviral shRNA infection, we observe changes in nucleolar morphology and function reminiscent of those seen in cells derived from *Arf* -/- embryos, strongly supporting the hypothesis of a dynamic nucleolar growth-suppression pathway

involving Arf. In support of this contention, a recent report () has demonstrated that selective disruption of the nucleolus by either UV radiation or a number of “stress” responses induces cell-cycle arrest and markedly enhances p53 stability. Furthermore, other groups have reported that overexpression of two large subunit ribosomal proteins (L11 and L23) can also stabilize p53 by binding and inactivating mdm2 in a manner similar to Arf. While we did not observe any gross disruption of nucleoli in cells either lacking or overexpressing Arf, we did observe numerous qualitative changes in the proteome of *Arf* -/- nucleoli, indicating that Arf might play a vital role in determining the composition of nucleoli (data not shown).

Numerous p53-independent functions have also been ascribed to Arf (), for example binding to and negatively regulating the activity of several pro-growth transcription factors such as myc and Foxm1b as well as interfering with nucle(ol)ar protein trafficking, either through sequestration of key ribosomal biogenesis factors (such as nucleophosmin) () or through enhanced sumoylation(). In addition, our laboratory (and others) have shown a direct interaction between Arf and nucleophosmin, and recent data from several groups including ours has indicated that such an interaction may be inhibitory towards nucleophosmin’s ability to promote ribosome biogenesis ().

While the mechanism and nature of such inhibition is still unclear, our data are consistent with a “thermostat” function for Arf, in that small changes in the abundance of the protein affect its binding partners in such a way as to either dampen or enhance ribosomal assembly and export, and ultimately lead to global changes in protein synthesis. One can envision a model whereby cells receiving inappropriate growth-promoting cues through mitogenic pathways such as PI3K-Akt-mTOR transiently increase production of Arf, which then negatively feeds back on

ribosomal biogenesis and protein synthesis, thereby dampening the effects of the inappropriate signal, and restoring homeostasis. Disruption of this exquisite control, then, would render cells highly susceptible to oncogenic signals, and may prove a selective advantage to premalignant cells. In support of this hypothesis, a recent study on methylation of key loci involved in colorectal carcinogenesis demonstrated that ~85% of the adenomas (pre-malignant lesions) isolated from patients with sporadic colorectal cancer demonstrated abnormalities at the p14^{ARF} locus. () In addition, mutations in the Ink4A/Arf locus are commonly observed in sporadic WHO grade II astrocytoma which often will progress toward high-grade glioblastoma over a number of years, whereas primary glioblastoma is less likely to harbor mutations in this locus. ()

Para on why our findings are different than Chuck's: eg he used grossly overexpressed Arf, we saw changes using physiological levels.

Final para commenting on how Arf may regulate all steps of ribosomal biogenesis including rRNA synthesis (possibly via indirect control of p53) and shuttling through NPM.

Our findings represent a novel and important role for Arf in maintaining protein synthetic homeostasis in non-malignant cells. While the precise details of how this may be achieved still remain elusive, understanding how the nucleolus (and by extension, Arf) couples the disparate requirements of proliferation, growth and ribosomal biogenesis through interactions with proteins like p53, mdm2, and nucleophosmin will deepen our knowledge of tumor suppressor mechanisms and may provide novel therapeutic targets for malignancy and other disease states where protein synthesis may be dysregulated.

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FIGURE LEGENDS

Figure 1: Disruption of p¹⁹ARF enhances protein synthesis independent of cellular proliferation. (A) Total radioactivity incorporated after ³⁵S-methionine pulse. Cells were starved of methionine and cysteine for 30 minutes prior to addition of label for the indicated times, followed by lysis, TCA precipitation of proteins, and liquid scintillation counting. (B) Growth curve of wildtype and *Arf* -/- MEFs. Equal number of cells (1×10^5) were plated in triplicate at day 0, and then trypsinized and counted via hemocytometer at various time points . (C) Cytoplasmic ribosome profiles from wildtype and *Arf* -/- MEFs. Cycloheximide (50 µg/ml) was added for 10 minutes prior to lysis and ultracentrifugation of cleared lysate on 10-40% sucrose gradients. Graph depicts A₂₅₄ of ribosomal subunits over increasing sucrose density.

Figure 2: Loss of p19^{ARF} results in nucleolar morphological changes. (A) AgNOR stain of representative wildtype and *Arf* -/- MEFs (x400). Notice the increase in number and irregularity of the AgNORs in the *Arf* -/- cells. (B) Ultrastructural features of nuclei from wildtype and *Arf* -/- MEFs. Top row: 3000x magnification. Bottom row: 7000x magnification. (C) Quantification of AgNOR indices from (A). Left panel: Number of AgNORs per nucleus (n=100). Right panel: Total nucleolar area (in μm^2) per nucleus as determined by histomorphometric analysis (n=100).

*= p<<0.01

Figure 3: Acute depletion of p19^{ARF} results in nucleolar morphological and functional changes reminiscent of *Arf* -/- cells. (A) Confirmation of p19^{ARF} knockdown in wildtype MEFs 96 hours post-infection with lentiviral shRNA constructs (B) AgNOR staining of representative MEFs infected with control (scrambled) or p19^{ARF}-specific shRNA virus (x200). (C) Quantification of AgNOR indices. Left panel: Number of AgNORs per nucleus (n=100). Right panel: Total nucleolar area (in μm^2) per nucleus as determined by histomorphometric analysis (n=100). *= p<<0.01 (D) Total radioactivity incorporated after ^{35}S -methionine pulse. Cells were starved of methionine and cysteine for 30 minutes prior to addition of label for the indicated times, followed by lysis, TCA precipitation of proteins, and liquid scintillation counting. (E) Cytoplasmic ribosome profiles from MEFs infected with control (scrambled) or ArfshRNA virus. Cycloheximide (50 $\mu\text{g}/\text{ml}$) was added for 10 minutes prior to lysis and ultracentrifugation of cleared lysate on 10-40% sucrose gradients. Graph depicts A₂₅₄ of ribosomal subunits over increasing sucrose density.

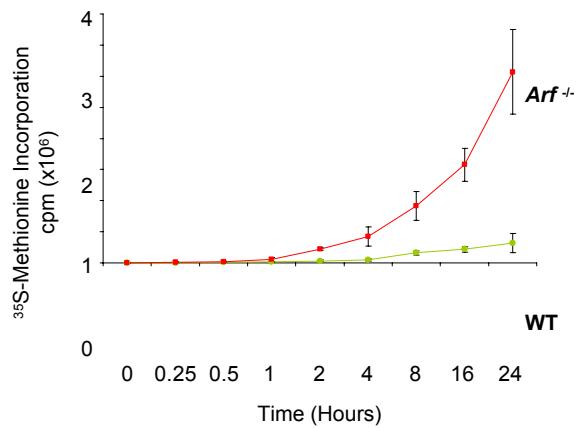
Figure 4: Loss of p19^{ARF} has functional consequences on osteoclast biology. (A) Representative TRAP-stain of equal numbers of bone marrow-derived macrophages following three days of treatment with M-CSF and RANK ligand reveals an increase in multi-nucleated osteoclasts formed from *Arf* -/- precursors. (B) Graph depicting increase in TRAP-positive osteoclasts with greater than five nuclei derived from *Arf* -/- bone marrow. * = p=0.01 (C) TRAP solution assay of equal numbers of TRAP-positive cells. Cells from either wild type (day 4 post-RANKL addition) or *Arf* -/- (day 3 post-RANKL addition) precursors were lysed and incubated in a colorimetric assay with *p*-nitrophenyl phosphate, a substrate for TRAP. Graph

depicts absorbance at 405 nm. * $=p=0.01$ (D) BrdU incorporation by wild type and *Arf* $-/-$ macrophages. No significant difference in proliferation rates were noted.

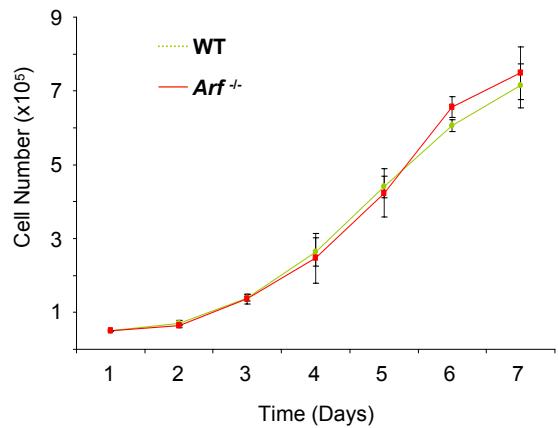
Figure 5: Arf exerts its effects through control of rRNA synthesis, ribosomal biogenesis and ribosomal export (A) Western blot demonstrating that *Arf* $-/-$ MEFs do not have alterations in the levels of nucleolar proteins NPM and ribosomal protein L5. (B) Serial NPM immunoprecipitation. Wildtype cells were lysed in Tween-20 buffer, and serially immunoprecipitated with mouse anti-NPM five times. The final supernatant was concentrated and included as a control for non-NPM binding proteins. LC- light chain (C) Relative abundance of the primary 47S rRNA transcript. Total RNA was collected from equal number of asynchronously dividing cells, and quantitative realtime RT-PCR performed with a primer specific to the mouse 47S transcript. (D) 3 H-uridine rRNA incorporation by asynchronously dividing cells. Wildtype and *Arf* $-/-$ cells were pulsed with labeled uridine for 30 minutes followed by chase in cold media for the indicated times. Total RNA was isolated, loaded onto formaldehyde-containing agarose gels, and transferred to membranes for autoradiography. (E) Nuclear-cytoplasmic fractionation of 3 H-labelled methylmethionine pulse-chase. Equal number of cells were pulsed with 3 H-methylmethionine and chased in cold methionine containing media for the indicated times. Total RNA was isolated from nuclear and cytoplasmic fractions and subjected to autoradiography. (F) Cytoplasmic fractions from the indicated times were also subjected to liquid scintillation counting to obtain a quantitative estimate of total cytoplasmic rRNA.

Figure 1. Apicelli et al.

a



b



c

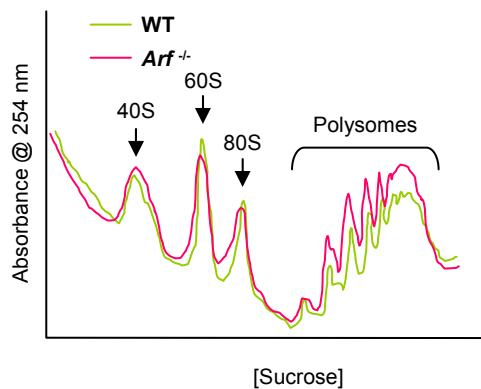
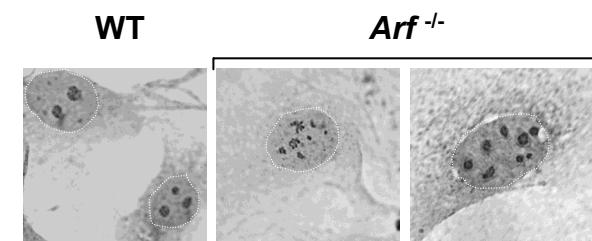
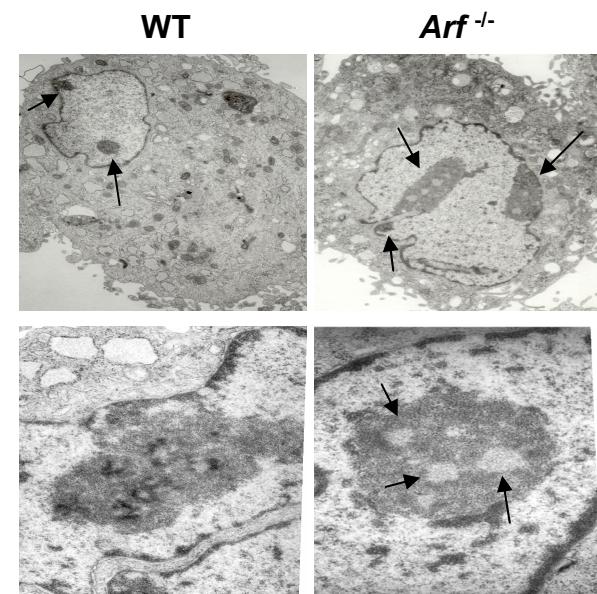


Figure 2. Apicelli et al.

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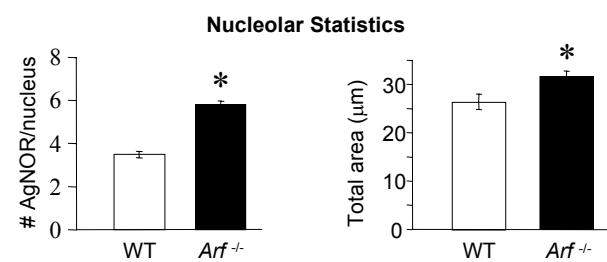
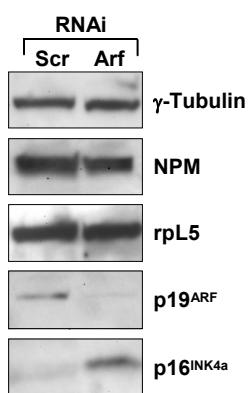


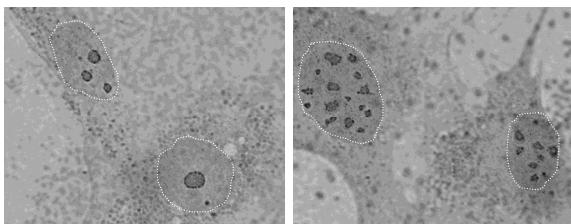
Figure 3. Apicelli et al.

a



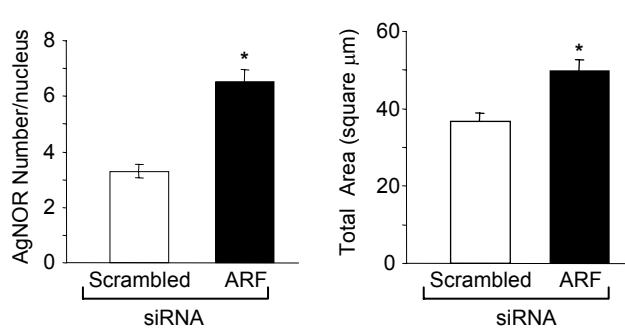
b

Scrambled siRNA ARF siRNA

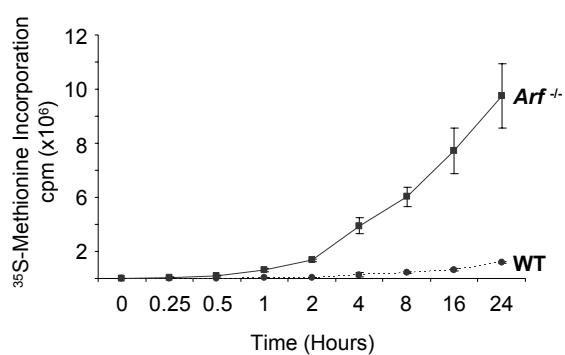


c

Nucleolar Statistics



d



e

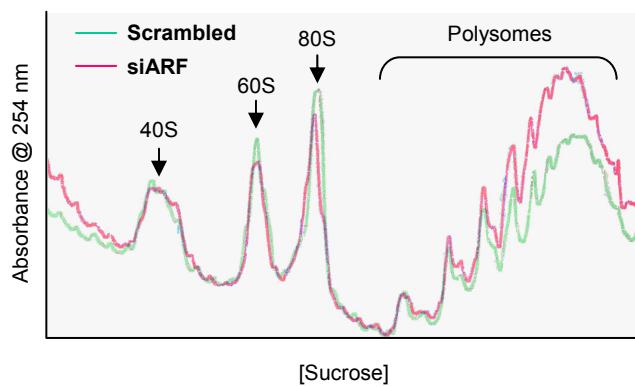


Figure 4. Apicelli et al.

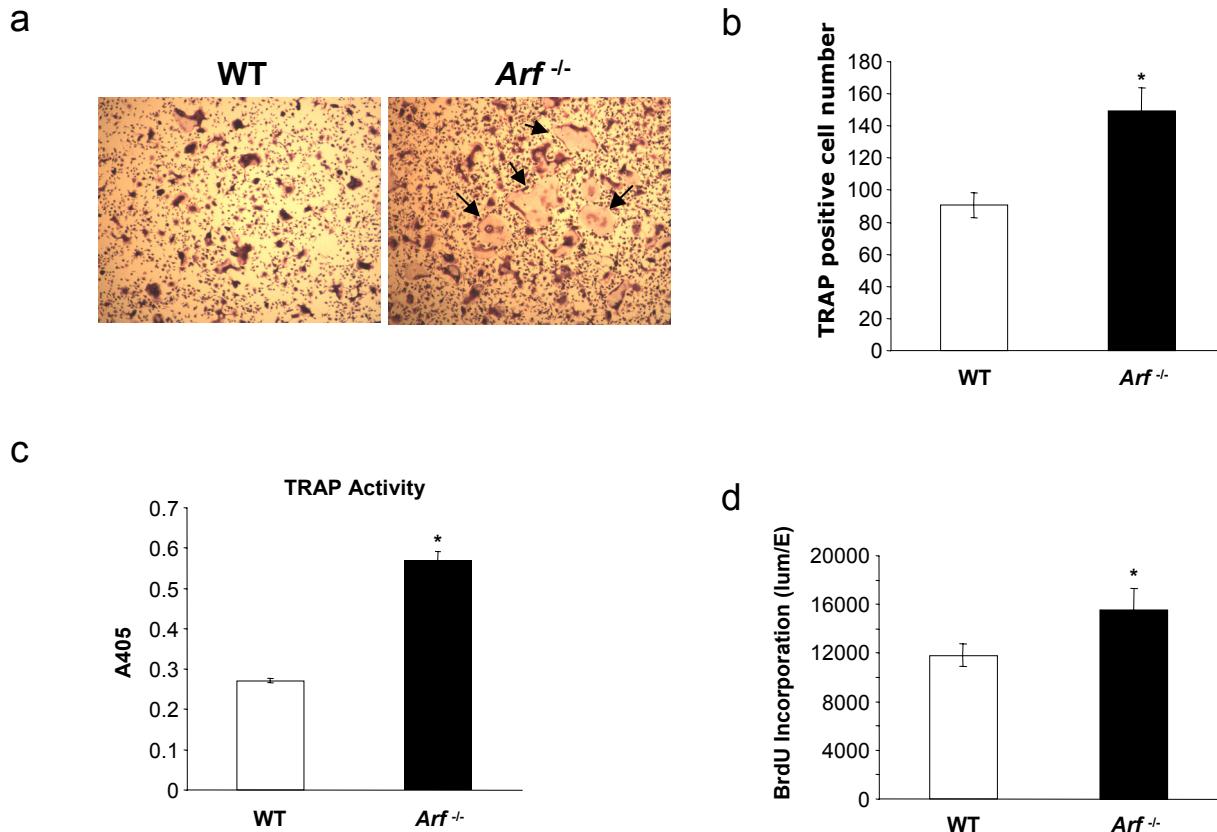
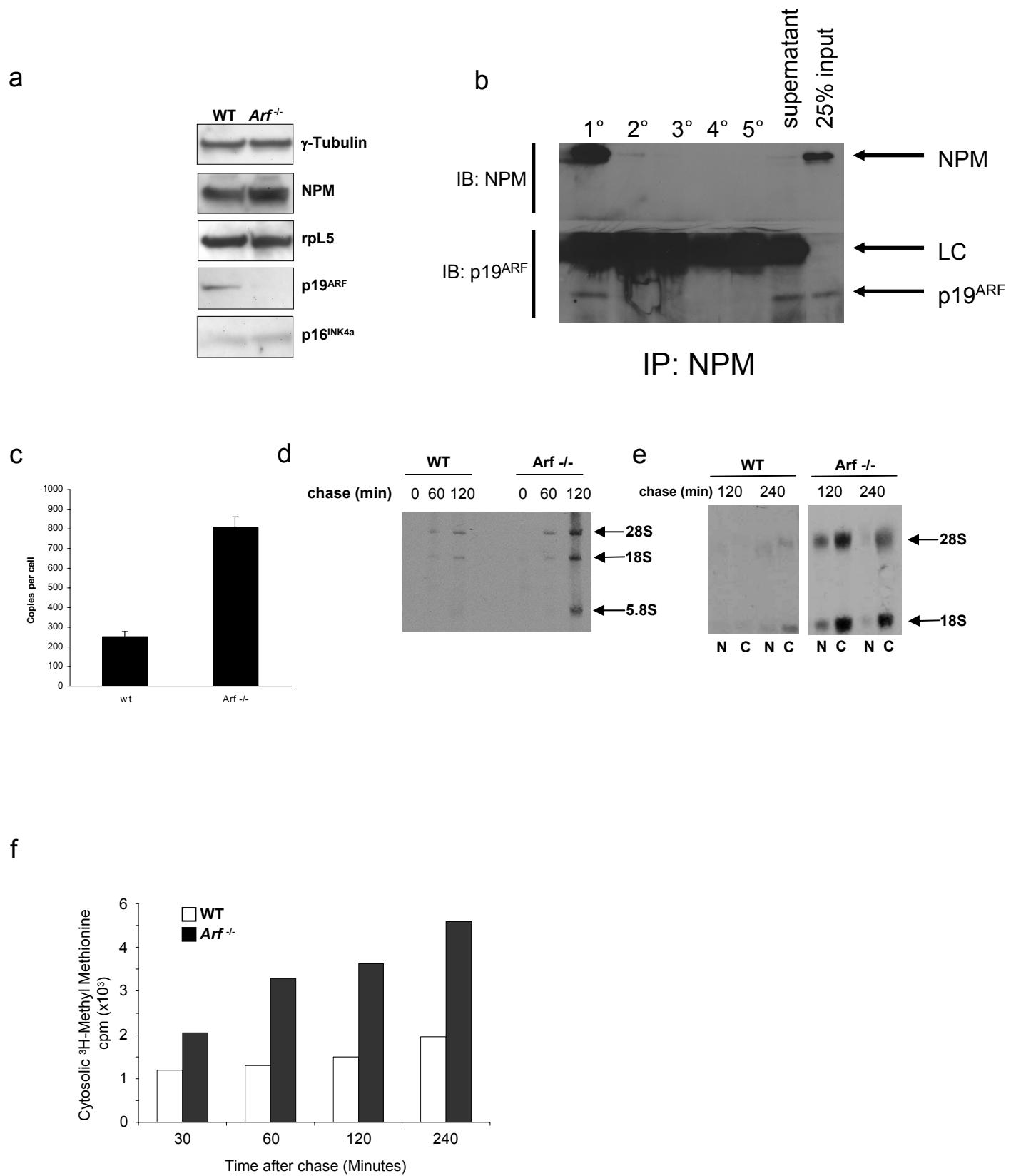


Figure 5. Apicelli et al.



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Abstracts:

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